THE EFFECT OF NITRATE AND AMMONIUM IONS ON THE METABOLISM AND GROWTH OF THE TOMATO PLANT, *LYCOPERSICUM ESCULENTUM* Mill.

A Thesis submitted to the University of Adelaide
for the Degree of Doctor of Philosophy.
by

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Degree of Doctor of Philosophy.

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H.W. Woolhouse.

CONTENTS

PREFACE

In the introductory discussion to this thesis the present evidence conderning the process of nitrate assimilation in the major plant groups is examined.

Chapter I describes experiments concerned with the role of ascorbic acid in nitrate assimilation in young tomato plants.

Chapter II describes experiments concerning various aspects of difference in growth between plants receiving nitrate and ammonium nitrogen.

Chapter III describes experiments on the phosphate metabolism of shoots of nitrate and ammonia grown plants. The experiments are discussed in relation to an hypothesis attempting to account for the differences in growth between plants receiving nitrate and plants receiving ammonium nitrogen.

I N T R O D U C T I O N

The capacity to assimilate nitrogen in the form of nitrate into the proteins and other constituents of the cell is a property of a wide range of plants and micro-organisms. There is a tendency to regard biochemical reactions as basically similar from one organism to the next. It is quite clear, however, that for technical reasons the type of experiments which have been carried out in order to establish metabolic reaction sequences differ between different types of organisms. For example, convenience of experiment has resulted in a large majority of the flashing-light experiments of photosynthesis being carried out with uni-cellular algae and aquatic plants. Such a state of affairs inevitably results in a conditioning of the information on the metabolism of a group of organisms. This situation is well exemplified in the literature on nitrate assimilation, and for this reason it is perhaps useful to examine the rapid expansion in this field during the past five years, from a comparative point of view, between the different groups of organisms.

MICRO-ORGANISMS:

If an organism reduces nitrate to ammonia, which can then enter into organic combination, one might

expect the growth and composition of the organisms when grown with ammonia as the nitrogen source would not differ from the growth and composition on nitrate, unless certain definable factors were involved:-

(1) The removal of electrons from some pool involved in the reduction of NO_3^- were vital to the redox potential at a particular site(s) in the cell where certain reactions occur.

A probable example of this situation is the denitrifying bacterium Micrococcus denitrificans, which under anaerobic conditions will grow only if nitrate is present in the medium. The nitrate consumed, however, is all accountable as N_2 or N_2O; none of the nitrate nitrogen appears in the components of the cell, and it is reasonable to suppose that the function of the nitrate is in this case as an essential electron acceptor.

(2) Removal of electrons by nitrate may bring about changes in, or stimulated growth of, the organism, although the nitrate is not essential for the growth.

Thus certain strains of Escherischia coli, under anaerobic conditions, are able to ferment glucose in the absence of nitrate, lactate and succinate being the main products of glucose break-down. Under similar conditions, but with the addition of potassium nitrate, acetic acid and carbon dioxide are the main products of

glucose fermentation which accumulate. (Revallier-Warffemius, 1955). Ninety-three percent of the nitrate reduced was converted to nitrite but not further reduced; it may be therefore that the removal of electrons, whilst not essential to the growth of the organism is bringing about a modification in the products of fermentation. It may be however, that the primary effect of nitrate on the cell is on systems other than glycolysis. No data are available, but it would be of interest to know the actual dry matter production in these experiments. Of further interest would be to know whether other artificial electron acceptors could bring about similar changes in the products of fermentation, to those evoked by nitrate.

(3) Nitrate, on reduction, might give rise to nitrogen containing metabolites of significance in metabolism, but which could not be so readily formed from ammonia, or some other primary nitrogen source at the same level of reduction.

Thus, for example, reduction of nitrate to hydroxylamine and subsequent reaction of this product with a keto acid to form an oxime which could then be further reduced, might result in the formation of a particular amino-acid "family", (concept exemplified in the work of Roberts et al. 1955,) which could not be so readily formed from ammonia. Although Meyer and Schulze 1884, suggested -C=NOH as the primary organic grouping formed

during nitrate assimilation, there are still only occasional reports of the formation of oximes or of their reduction in living cells, and nothing is known of their significance, if any, in bacterial metabolism.

As will be seen later however, there is some evidence for oxime metabolism in other plant groups, and it may be that technical difficulties, e.g. toxicity and instability of the oximes, difficulty in purifying them free from traces of hydroxylamine, inability to detect appropriate co-factors or the extreme lability of the oxime-reducing enzymes might account for the present uncertainty as to whether or not they are concerned in metabolism. The possibility exists, of course, of a variety of organo-nitro compounds other than the above-mentioned oximes being of significance in nitrate assimilation but there is only scant evidence for such intermediates.

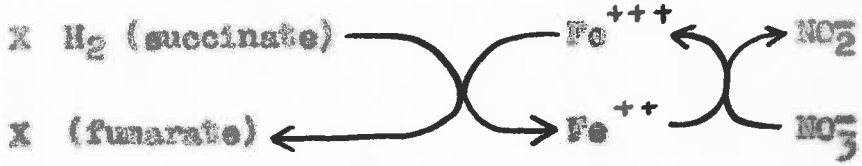
(4) There may be different adaptive responses to the two substrates, nitrate and ammonium.

That this is likely to be so is readily demonstrated with a wide range of micro-organisms, the nitrate reducing systems being absent from the cell when grown on ammonia as the nitrogen source but being developed on exposure to nitrate.

(5) In the course of passage of energy, electrons, from donor to nitrate oneway have a coupling of reactions so that energy is "channelled" in specific directions

and hence the growth of the organism is modified.

In order to exemplify situation (5) it is convenient to turn to a further aspect of nitrate reduction, i.e., the nature of the electron pathway between donor and acceptor. Sato and Egami, 1949, showed spectroscopically that under anaerobic conditions a cytochrome b_1 extracted from E. coli, which resembled heart muscle cytochrome b, became oxidised in the presence of nitrate. The nitrate was shown to be reduced to nitrite and succinate was able to serve as an electron donor. In general terms the reaction was represented diagrammatically by the scheme:-



the valency change in the iron being a postulate in the model, since it was not directly demonstrated. Sato 1955, using a sensitive double beam spectrophotometer (Chance, 1951) was able to make similar observations on turbid suspensions of intact cells of E. coli. Addition of nitrate to a concentration of 1.5×10^{-5} M resulted in oxidation of the cytochromes within thirty seconds. When the nitrate became used up the cytochromes again became reduced. Both reduction of nitrate and oxidation of the cytochrome were completely inhibited by 10^{-3} M potassium cyanide. Supporting evidence for the involve-

ment of the cytochrome in the nitrate reducing system came from the fact that the cytochrome b_1 oxidation-nitrate reduction was 70% inhibited by 2-heptyl-4-hydroxyquinoline-N-oxide, (HOQNO), an inhibitor shown by Lightbown and Jackson, 1954, to be a specific inhibitor of the oxidation of cytochrome b_1. Many aspects of this system require further investigation; thus in Sato's work, increase in HOQNO concentration to 5×10^{-4} M resulted in no further inhibition of nitrate reduction. It is possible that the molybdo-flavo protein reducing nitrate, isolated from E. coli by Nicholas and Mason, 1955, might account for the 30% of HOQNO insensitive nitrate reduction in this organism. This suggestion gains some support from the observation that in two organisms known to have a well developed molybdenum-nitrate reductase system the cytochromes are unaffected by the addition of nitrate under anaerobic conditions. It is alternatively possible, however, that this molybdenum containing enzyme is an integral part of the cytochrome sequence. This point will be referred to later in relation to work on higher plants. Considerable controversy has arisen over the identity, or otherwise, of cytochrome b_1 and nitrate reductase. They may be distinct enzymes, or different prosthetic groups bound to a common protein. The more significant problem is whether or not the cytochrome naturally and normally occupies a position in the pathway

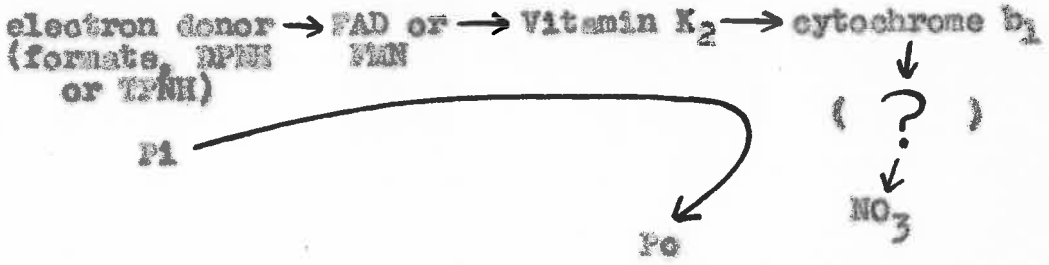
of electron flow to the nitrate.

Wainwright, 1955, investigated the nitrate reductase activity of a particulate acetone powder preparation from E. coli using reduced diphosphopyridine nucleotide (DPNH) and reduced triphosphopyridine nucleotide (TPNH) as electron donors in the presence of flavine adenine dinucleotide (FAD). The preparation showed a negligible nitrate reductase activity unless a concentrated aqueous extract of the cells was added to the reaction mixture. Of a range of possible co-factors tried only menadione, 2-methyl-naphthoquinone-(1:4), with fifteen minutes anaerobic pre-incubation and 1.25×10^{-3} M ferrous iron, stimulated nitrate reductase activity. It was suggested that the menadione served as a precursor for vitamin K_2 which is known to occur naturally in E. coli and many other bacteria.

For E. coli it seems that components similar to the electron transport chain of respiration appear to be associated with the reduction of nitrate. This similarity led Takahashi et al., 1956, to see whether there was any formation of organo-phosphate compounds concomitant with nitrate reduction. Using E. coli under anaerobic conditions, with formate as electron donor, these authors were able to show a significant enhancement of p^{32} incorporation following addition of nitrate.

Thus summarising the evidence for the reduction

of nitrate by E. coli we may write:-



From the preceding discussion it is clear that a simplification of this type can in no way account for the many varied results obtained with other micro-organisms. The generalisation is formulated here for purposes of comparison with information on other plant groups to be discussed later.

FUNGI.

Studies on nitrate reduction and assimilation up to 1945 have been reviewed by Burstrom, 1945. Subsequent to this date there have been considerable advances particularly from the application of the bio-chemical-genetic techniques of Beadle and Tatum to the ascomycete, Neurospora crassa.

THE INORGANIC PATHWAY IN N. crassa.

A series of enzymes have been isolated from Neurospora capable of reducing nitrate by a sequence of two-electron transfers, to ammonia, the sequence of intermediates being:-

$NO_3^- \longrightarrow NO_2^- \longrightarrow HNO \longrightarrow NH_2OH \longrightarrow NH_3$

It is of interest to see what is known of each of these reduction steps and the enzymes catalysing them before attempting to relate them to the metabolism of the whole organism.

NITRATE REDUCTASE: Evans and Nason, 1952, isolated and partially purified an enzyme from N. crassa which catalysed the reduction of nitrate to nitrite using DPNH or TPNH as an electron donor, the TPNH being some four times more effective in vitro than the DPNH. The stoichiometry of the reaction was shown to be

$$TPNH + H^+ + NO_3^- \longrightarrow TPN^+ + NO_2^- + H_2O$$

Subsequently Nason and Evans, 1953, showed the enzyme to be adaptively formed in the presence of nitrate or nitrite, but not ammonia or alanine, and to require FAD for its activity. Inhibition of the enzyme by such substances as azide, cyanide and O-phenanthroline suggested a metal requirement for activity and led to an examination of the effects of metal nutrient deficiencies on the enzyme activity, Nicholas et al., 1953, 1954a. From this work the evidence of Steinberg, 1937, using Aspergillus niger, that molybdenum was essential for nitrate reduction was confirmed. A direct relationship between molybdenum content of the felts and nitrate reductase activity was established. A curious feature of these nutritional studies was that deficiencies of calcium,

iron, copper, and zinc resulted in an enhanced nitrate reductase activity, a result for which no adequate explanation has been offered. Nason and Evans, 1953, showed p-chlor-mercuribenzoate 10^{-4} M. (PCMB) to be a strong inhibitor of nitrate reductase activity suggesting that -SH groups were an essential component of the enzyme. Subsequently Nicholas and Nason, 1954b, found that PCMB was a strong inhibitor (100%) of nitrate reductase activity if TPNH was used as the electron donor, but using reduced flavine mono-nucleotide ($FMNH_2$) only a feeble (25%) inhibition of activity was observed, thus suggesting that -SH groups were essential for the attachment of the TPNH to the enzyme and that TPNH preceded FMN (or FAD) in the electron transporting sequence. This enzyme preparation, when dialysed free from molybdenum, lost the capacity for nitrate reduction, but was still able to catalyse the reduction of FAD by TPNH. Addition of molybdenum restored the ability of the preparation to reduce nitrate, so that the effect of the molybdenum appears to be between the FAD and the nitrate. Summarising:-

$$TPNH \longrightarrow FAD \text{ (or FMN)} \longrightarrow Mo \longrightarrow NO_3$$

Kinsky and McElroy, 1958, carried out an extensive purification of a nitrate reductase from N. crassa, and found that during purification the increase in specific activity of enzyme was paralleled by an increase in TPNH-cytochrome C reductase activity for the same pre-

paration. Nitrate reductase activity was absent from ammonia grown felts and TPNH-cytochrome C reductase showed only 20% of the activity developed when nitrate was present in the medium. It would be of great interest to know whether this TPNH-cytochrome C reductase adaptively formed in the presence of nitrate requires molybdenum for its functioning as does the nitrate reductase.

NITRITE REDUCTASE: Nason and Evans, 1953, and Nason et al., 1954, isolated a metallo-flavo protein from N. crassa catalysing the reduction of nitrite, using TPNH as an electron donor. Medina and Nicholas, 1957a, provided evidence for two metals, copper and iron, as components of the enzyme. These workers found DPNH to be an electron donor. As with most studies on these enzymes there are no reports of attempts to determine the transhydrogenase activity of the preparations, so that the reduced nucleotide specificity is uncertain. Nicholas, 1958, identified cytochromes b and c1, in the purified enzyme ($\lambda_{max.}$ 553 and 562 m μ) reduction of which by DPNH was enhanced by addition of FAD suggesting that FAD preceded the cytochromes in the electron transporting chain. Addition of nitrite resulted in a re-oxidation of the cytochromes. Cuprous chloride stimulated enzymic reduction of nitrite but not the reduction of

the cytochromes. Addition of FAD did not enhance the effect of the cuprous ion. Inhibition of the system by uncoupling agents suggested that a phosphorylation was involved in nitrite reduction. Diagramatically the sequence of events in nitrite reduction suggested by Nicholas may be summarised as:-

Thus in the case of nitrite reductase from M. crassa, evidence suggests a situation of the general type refered to in E. coli whereby the electron-transporting system associated with nitrite reduction is coupled to an energy fixing (phosphorylating) system.

HYPONITRITE REDUCTASE:

Baudisch, 1923, detected the formation of potassium nitrosyl K(NO) from nitrate in the presence of sunlight and suggested that in living cells this might react with an aldehyde to form a hydroxamic acid, subsequent reduction of such a product would lead to the formation of an aldoxime and eventually an amide.

The instability of hyponitrous acid and its salts delayed progress in the study of this compound as a possible intermediate in nitrate reduction. Medina

and Nicholas, 1957b, isolated from N. crassa a metal-requiring flavoprotein which catalysed the reduction of hyponitrite to ammonia. Iron and copper were shown to be essential for enzyme activity; DPNH served as an electron donor. The further study of hyponitrite reducing systems from fungi, has not yet been reported.

HYDROXYLAMINE REDUCTASE:

Meyer and Schulz, 1884, suggested that hydroxylamine might be an intermediate in the reduction of nitrate, but the extreme toxicity of this compound to most organisms proved an obstacle to its further study. Wirth and Nord, 1942, and 1943, and Nord and Mull, 1945, demonstrated the presence of hydroxylamine in Fusarium grown with nitrate as a source of nitrogen. The first enzymic studies with fungi are those of Zucker and Mason, 1954, and 1955a, who isolated a soluble enzyme from N. crassa catalysing the reduction of hydroxylamine to ammonia.

The maximum activity using TPNH as electron donor was twice that obtained using DPNH. As mentioned in connection with other reductases, however, no measurements of transhydrogenase activity were made. The stoichiometry of the reaction was found to be:-

$TPNH + H^+ + NH_2OH \rightarrow TPN^+ + NH_3 + H_2O$. FAD but not FMN enhanced the activity of the enzyme. Inhibition by che-

lating agents suggested a metal requirement for the enzyme activity; the metal was identified by Nicholas, 1957, to be manganese.

GENETIC EVIDENCE:

The problem of the extent to which the enzymes of the "inorganic pathway" contribute to the total nitrate reduction by any organism faces the classical difficulty of bridging the gap between the behaviour of isolated enzyme systems and their normal in vivo functioning. Further, no single method is as yet available for deciding the relative contribution of alternative biochemical sequences, so that one is compelled to assess the evidence from as many different sources as possible. In the case of nitrate assimilation, genetic studies, almost all using N. crassa, have served at least to emphasise the complexity of the situation.

De la Haba, 1950, obtained three mutants of N. crassa which were able to use nitrate or nitrite as a nitrogen source, but not ammonia, a situation not compatible with the "inorganic pathway". However, it must be noted that in De la Haba's report, and indeed in many of the published papers dealing with this problem, the genetic data quoted is insufficient for the reader to decide whether the mutants studied are of the simple one factor type. In order to explain this he postulated the existence of an

"organo-pathway" whereby $R-NO_2$ was reduced to $R-NH_2$ with subsequent transamination to form amino acids.

Silver and McElroy, 1954, obtained other Neurospora mutants which were unable to use nitrate, but which grew well on nitrite or ammonia. Felts of such mutants were found to contain no nitrate reductase suggesting that this enzyme occupied a position in nitrate reduction. Further evidence for the importance of nitrate reductase came from a mutant which showed a varied capacity for growth, from no growth at pH 5.0 to a maximum at pH 7.0. This growth curve was closely paralleled by the pH-nitrate reductase activity curve for an in vitro preparation of the enzyme. If the relationship between these two curves is other than coincidental it suggests that the nitrate reductase in the living cell is directly exposed to the ambient solution, being located either at the cell surface, or in some "free space" deeper in the cell. There exists the possibility of course that the mutation has affected some other less specific factor than the enzyme, for example in the case of nitrate reductase, a reduced electron donor synthesis. Silver and McElroy investigated three TPNH generating systems, glutamic dehydrogenase, 6-phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase in this connection. All showed activity comparable with the wild type, suggesting that the genetic effect must be on the apoenzyme.

Genetic studies on nitrite reductase in Neurospora yielded different results from those obtained for nitrate reductase. Mutants were found which were unable to use nitrite as a nitrogen source. Homogenates from these mutants, however, showed an appreciable nitrite reductase activity. One may be observing an in vivo deficiency of a co-factor, suchas an electron donor, or in extracting the cells certain moieties may be modified so as to become catalysts of nitrite reduction. Again the extraction process may result in the release of stimulators or neutralisation of enzyme inhibitors resulting in the nitrite reductase activity found in vitro. It would be of considerable interest to know whether the cytochromes associated with nitrite reductase show any signs of abnormality in the non-nitrite reducing mutants, as compared with wild-type. Two of the mutants found by Silver and McElroy to be unable to reduce nitrite, grew normally on nitrite following the addition of pyridoxal phosphate to the culture medium. Such mutants, when grown under varying concentrations of pyridoxal phosphate, developed a proportional nitrite reductase activity. Nitrate reductase activity was not affected by pyridoxal phosphate concentration. Under certain conditions these mutants were able to grow on ammonia without addition of pyridoxal phosphate. From these observations the hypothesis was made that pyridoxal phosphate was essential

for nitrite reduction according to the following scheme:-

$NO_3 \rightarrow NO_2 \rightarrow HNO \rightarrow NH_2OH$ + pyridoxal phosphate $\rightarrow$ oxime pyridoxal phosphate $\rightarrow$ pyridoxamine phosphate.

Presumably a pyridoxamine phosphate transaminase of the type described by Beechey and Happold, 1957, for animal tissues, could account for the formation of amino acids and the regeneration of pyridoxal phosphate. This scheme takes no account of the question of why nitrite should be the intermediate which accumulates. Zucker and Nason, 1955b, isolated enzymes from Neurospora which reduced m-dinitrobenzene to m-nitrophenyl hydroxylamine and thereafter to m-nitroaniline. The enzymes involved differ from nitrite and hydroxylamine reductases in not being adaptively formed in response to nitrate. It is doubtful whether these enzymes are specific for m-dinitrobenzene since m-nitroaniline, once formed, accumulates, and does not appear to be further metabolised by Neurospora. It would be of interest to know whether they are able to catalyse the reduction of nitro derivatives of pyridoxal phosphate. It must be noted in discussing this work, that it remains quite possible that the effects of pyridoxal phosphate are indirect and bear little relation to the schemes so far suggested.

A final isolated fact which serves to indicate further the complexity of the problem of nitrate reduction

may be cited from the genetical studies of Silver and McElroy, 1954. A single mutant was obtained which was unable to use either nitrate or nitrite as a source of nitrogen but if a "catalytic" amount of ammonia was added to the medium, normal growth resulted on nitrate or nitrite.

UTILISATION AND IDENTIFICATION OF INTERMEDIATES:

This problem will be examined more thoroughly in relation to higher plant studies. Briefly it may be said that doubts have been expressed about the supposed intermediates in nitrate reduction on the grounds that they can rarely be isolated from living tissues, and when fed to various organisms as sources of nitrogen often prove highly toxic. Steinberg, 1953, using Aspergillus niger and Nicotiana tabacum showed that sodium nitro-hydroxylaminate was far more effective than hydroxylamine, hyponitrite or nitrite in supporting growth (dry weight production) and protein synthesis, and argued that this compound was therefore more likely to be an intermediate in nitrate reduction. There are as yet no reports of the isolation of a nitrohydroxylaminate reducing enzyme or of attempts to use this compound as a source of nitrogen for Neurospora.

NITRATE ASSIMILATION BY ALGAE:

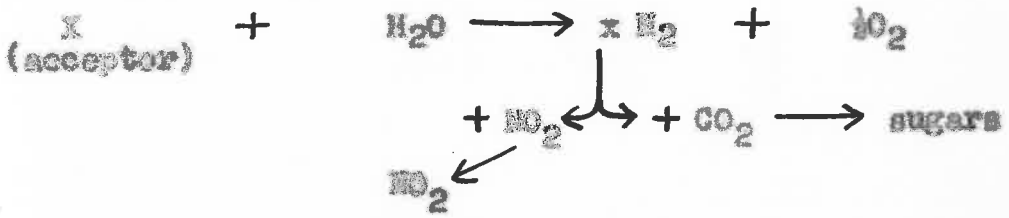
Although the genetic tool does not readily

lend itself to algal studies, other factors, and in particular, the relative ease of culture of the unicellular fresh water forms, has made them popular for physiological studies. Work on nitrate reduction reflects the culture methods available; thus more is known of the physiological aspects of nitrate reduction, particularly its relation to photosynthesis and respiration than is the case with fungi or higher plants. Detailed work on biochemical pathways and enzyme mechanisms, on the other hand, is relatively lacking.

LIGHT IN RELATION TO NITRATE REDUCTION:

Muenscher, 1923, showed that Chlorella cells growing in the dark were able to reduce and assimilate nitrate, a result which has been subsequently confirmed many times. Warburg and Negelein, 1920, also working with Chlorella, found a more rapid production of ammonia from nitrate in the light than in the dark over a two-hour period, an observation also confirmed by others, Myers, 1948, and Kok, 1951. The problem raised by these two observations is: are there at least two enzyme systems involved in the reduction of nitrate by Chlorella, one independent of light, and the other photoactivated, or is there a more rapid and perhaps more complete reduction of nitrate in the light resulting indirectly from the enhanced production of an accessory factor, e.g. a nitrate reductant, in the course of photosynthesis? Kok,

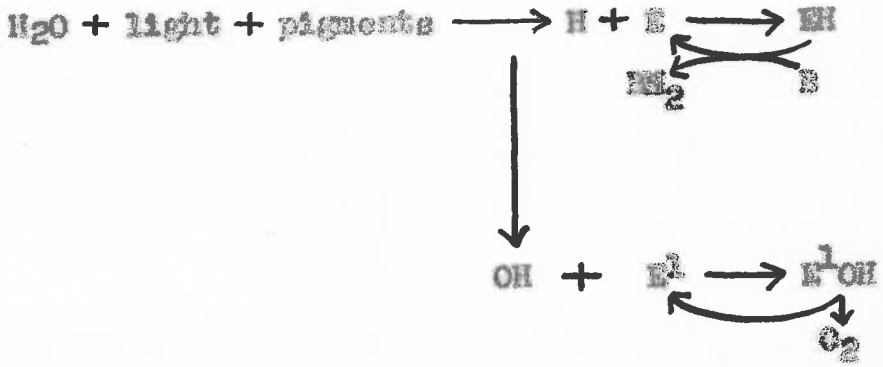
1952, Van Niel et al, 1953, and Van Overschot, 1955, provided evidence for the direct effect of light by showing that at low light intensities the presence of nitrate decreased carbon dioxide fixation by Chlorella suspensions. The magnitude of the effect varies markedly according to the experimental conditions employed by the different authors. It was postulated by Van Niel as early as 1941 that under these conditions nitrate was competing with carbon dioxide for the reductant generated in the course of the light reaction of photosynthesis, namely:



This still does not answer the question, of course, as to whether the same nitrate reducing enzyme(s) as is active in the dark is the one which competitively utilised the reductant generated during photosynthesis. This question is at present unanswered in the case of algal cells.

Under conditions of high light intensity at 25° C. the rate of evolution of oxygen is higher over a 2 hour period, from cell suspensions receiving 0.01M nitrate and 5% carbon dioxide than when carbon dioxide alone is present, Van Niel, 1953. Van Niel extended the hypothesis of photoreduction of nitrate to explain

this result as shown in the following scheme. The scheme has value in suggesting further experimental approaches to the problem though in fact it may well be an oversimplification.



It is supposed that at high light intensities the rate of photosynthesis is limited by the enzyme(s) transfering hydrogen to the acceptor B (CO_2 or its derivatives). In the presence of nitrate, however, a second H-acceptor B^1 transfering electrons to the nitrate might enhance the rate of regeneration of E from EH and hence the rate of photosynthesis and oxygen evolution might also be increased. This hypothesis is, however, difficult to reconcile with the observation that in the absence of carbon dioxide there is no evolution of oxygen from cells of Chlorella receiving nitrate, or at least only after a lag period of several hours during which photosynthetic fixation of carbon dioxide formed in the course of respiration could account for the oxygen evolved, Fan et al, 1943, Davies, 1953. It remains possible, though unlikely, that all of the oxygen evolved during the photolytic reduction of nitrate might be consumed in situ in the

course of respiration. It would seem that further work, probably with cell free extracts where the respiratory and photosynthetic systems could be separated, might be of use in resolving this controversial question of the photochemical reduction of nitrate by Chlorella.

The observed secretion of nitrite, e.g. Kessler, 1952, Bongers, 1956, into the culture medium of cells of Chlorella supplied with nitrate provides evidence for this compound as a product of nitrate reduction and also suggests a further possible explanation of the failure of nitrate to stimulate oxygen production in the absence of carbon dioxide. It could be that carbon dioxide is essential to the formation of a suitable carbon skeleton with which the products of nitrate reduction might react. It should be noted here however that Kessler 1952 and 1953a & b, found that the secretion of nitrite varied between different strains of algae and Bongers, 1956, showed a pronounced influence of pH of the culture medium on the secretion of nitrite by Chlorella. Bongers experiments were carried out using illuminated cell suspensions at 30° C and pH 7.5 under which conditions secretion of nitrite is high, how far such conditions correspond to the usual physiological conditions encountered by cells in the wild, is uncertain. Bongers further found that under the above-mentioned conditions Chlorella suspensions formed cell nitrogen when receiving carbon dioxide in the light but secreted ammonia into the medium when

carbon dioxide was excluded from the cultures; the influence of pH on ammonia secretion is not recorded. If the secreted nitrite and ammonia are derived from reduced nitrate, this finding of Bongers is not compatible with the previously mentioned diminished nitrate reduction in the absence of CO_2, Fen et al, 1943, Davies, 1953, unless a difference of algal strain or experimental conditions used by these workers accounts for the difference.

Onura, 1954, reported nitrate and nitrite reductase activity in cell free extracts of Chlorella, but the length of time for incubation, up to twenty hours, leaves some doubt as to whether bacteriostatic conditions were maintained.

Yamafugi et al, 1954, reported the presence of a pyruvic oxime reducing enzyme in cell free extracts of Scenedesmus. The conditions of experimentation are not, however, adequately described. Further work on the enzymology of nitrate reduction by algae is clearly needed.

Kessler, 1953b, and Proctor, 1957, have emphasised the considerable differences between the various algae in their response to different inorganic nitrogen sources, and the potential value of comparative studies is great, e.g. Kessler, 1956, has shown that the green alga Ankistrodesmus braunii can adaptively form hydrogenase which can be coupled to nitrite reduction. Kessler's studies on Ankistrodesmus resulted in the further interest-

ing finding that although increased oxygen production in the presence of nitrate in the light was dependent upon the presence of carbon dioxide, nitrite addition to all cell suspensions in the light resulted in an immediate increase in oxygen production even in the absence of carbon dioxide suggesting a direct photochemical reduction of nitrite but not of nitrate in this species. This nitrate reduction was inhibited by DNP suggesting a phosphorylation reaction to be involved. There is here an interesting comparison with the previously mentioned demonstration by Nicholas of a phosphorylation coupled to the reduction of nitrite in Neurospora.

MINERAL NUTRITION:

Despite the extensive literature on the mineral nutrition of unicellular algae there are relatively few papers concerning their metal requirements in relation to inorganic nitrogen assimilation. Arnon etal, 1955, and Ichioka et al, 1955, showed molybdenum to be essential for the growth of Scenedesmus suspensions and calculated the requirement for optimal growth to be of the order of 3,000 atoms of molybdenum per cell. If a reduced form of nitrogen, such as ammonium carbonate or urea was used the molybdenum requirement was greatly reduced, and the rather sweeping conclusion was drawn that the sole function of molybdenum in this species was in nitrate reduction. In contrast, Wolfe, 1954a & b,

working with the blue-green alga Anabaena cylindrica, found that in the absence of molybdenum the cells could reduce nitrate to ammonia but could not carry out the further assimilation of ammonia into protein and other cell constituents. It is clear that further work is needed on other metals and on comparisons between different algal groups in relation to nitrate assimilation.

RESPIRATION IN RELATION TO NITRATE ASSIMILATION:

Warburg and Negelein, 1920, showed that the addition of nitrate to Chlorella suspensions maintained in darkness resulted in an increase in the rate of respiration during the first hour, and a subsequent decline in the rate of gas exchange. Liberation of carbon dioxide increased more rapidly than oxygen consumption, the R.Q. rising from 1.1 to 1.6 in the first hour. From the third to the fifth hour the rate of evolution of carbon dioxide fell more rapidly than the fall in rate of oxygen consumption, the R.Q. falling to 1.2

It was postulated that if nitrate is reduced to ammonia according to the endergonic reaction:

$$NO_3^- + H_2O + 2H^+ \longrightarrow NH_4^+ + 2O_2$$

and this reaction be linked to the exergonic oxidation of sugars:

$$2O_2 + 2(CH_2O) \longrightarrow 2CO_2 + 2H_2O$$

then combining these two equations:

$$NO_3^- + 2H^+ + 2(CH_2O) \longrightarrow NH_4^+ + 2CO_2 + H_2O$$

Then for each molecule of nitrate reduced to ammonia,

there would be two molecules of carbon dioxide evolved without any corresponding consumption of oxygen. Thus it was argued that this "extra carbon dioxide" formed in excess of the volume of oxygen taken up was a consequence of this postulated coupling of carbohydrate oxidation to nitrate reduction. Much higher ratios, however, of "extra carbon dioxide" evolved: ammonia formed, were obtained than the 2 : 1 ratio predicted from equation (1). Warburg and Negelein suggested this discrepancy to be due to the relatively rapid assimilation into other compounds of some of the ammonia formed. As Burström, 1945, pointed out, however, even if a ratio of "extra carbon dioxide" evolved: ammonia formed, in conformity with equation (1), were obtained, it would not constitute a proof of a direct linking of carbohydrate oxidation to nitrate reduction. Yasagata, 1934, found that ammonium ions stimulated the rate of respiration of Aspergillus oryzae, an effect also demonstrable with unicellular algae; in this case, however, the R.Q. is lowered, the extent of the lowering being dependant on the nature of the carbon source. Subsequent work on the coupling of nitrate and ammonia assimilation to respiratory processes presents a very confusing situation largely because of the use of nitrogen starved cells as experimental material. e.g. Syrett, 1955, 1956a, b, c, 1953, Bongers, 1956, and Hattori, 1957. It has frequently been shown, e.g. Fogg et al., 1953, Bongers, 1956, that nitrogen starvation has profound

effects upon the gross composition of algal cells, a situation which must in turn reflect major modifications of the enzyme content and activity within the cells. Results obtained with this type of material may differ greatly from those obtained with more normally cultured cells, and are in consequence of somewhat limited value.

Syrett, 1955, extended the theory of Warburg and Negelein to suggest that, if unlike Warburg's experiments, ammonia did not accumulate in the cell, then one could expect the gas exchange from nitrate reduction to be superimposed on the exchange associated with reduction product assimilation. It was further argued that if nitrite and hydroxylamine were added, then the following general equations for linked carbohydrate oxidation should hold:

$$1\tfrac{1}{2}(CH_2O) + NO_2^- + 2H^+ \rightarrow NH_4^+ + 1\tfrac{1}{2}CO_2 + \tfrac{1}{2}H_2O$$

.....(ii)

$$\tfrac{1}{2}(CH_2O) + NH_2OH + H^+ \rightarrow NH_4^+ + \tfrac{1}{2}CO_2 + \tfrac{1}{2}H_2O$$

Thus the ratios of "extra carbon dioxide" evolved: nitrogen reduced should be 2:1, 1.5:1, and 0.5:1 for nitrate, nitrite and hydroxylamine respectively, after correcting for carbon dioxide evolution associated with ammonia assimilation. Using nitrogen-starved cells of Chlorella vulgaris approximate agreement with these ratios was found in the case of nitrate and nitrite. Addition of hydroxylamine, however, resulted in an excessive stimulation of oxygen consumption, probably a

consequence of "toxic" effects of this compound. Further corrections to these ratios are necessitated by the finding that a dark fixation of carbon dioxide, up to one third of the volume of carbon dioxide released during respiration, is known to occur following the addition of ammonia to nitrogen starved Chlorella suspensions. Addition of nitrate also enhanced carbon dioxide fixation, though to a lesser extent Syrett, 1956c. Reasoning that a correlation might be expected between respiratory activity and phosphorylation reactions of the cell, Syrett, 1958, measured levels of adenosine triphosphate (ATP) in cells of Chlorella in the dark following stimulation of respiration by ammonia and other compounds. Addition of ammonia resulted in an initial lowering of the ATP concentration. Subsequently however, the ATP level rose again, although the rate of respiration was still increasing. Unfortunately similar studies using nitrate were not recorded, it is however, likely that the level of ATP will be influenced by many factors and a simple correlation even if established would be difficult to interpret. From this brief reference to an extensive literature, it is seen that the linking of nitrate reduction and assimilation of reduction products, to respiratory processes is complex and unlikely to be capable of description in terms of generalised equations of the type originally postulated by Warburg and Negelein, 1920.

INORGANIC NITROGEN ASSIMILATION IN HIGHER PLANTS:

(1) Site in the plant.

Studies on micro-organisms are valuable in providing information on metabolism at the cell level, and information gained from such studies may provide useful pointers to similar systems in higher plant cells. There is another class of problems, however, unique to multicellular organisms; these are the problems of the specialisation of metabolic reactions in particular organs and tissues, and the way in which they are correlated in the growth of the whole plant.

Burstrom, 1945, discussed the question of the capacity of different plant organs to assimilate nitrate, and argued that the most conclusive evidence of the ability of a particular organ to assimilate nitrate is from measurements of nitrate consumption by the excised organ. Nitrate reduction in isolated leaves has frequently been shown, e.g. Kaleski, 1901, Mittrich, 1930, and Burstrom, 1943, in isolated leaf primordia and callus tissue in culture medium by White, 1939, and in isolated root systems by Postma, 1939, and subsequently many other workers. However, the fact that an excised organ has the capacity to reduce added nitrate need not imply that when attached to the plant it would normally be doing this. Thus,

excised roots are able to assimilate a variety of compounds as carbon sources, which probably never occur in the normal course of metabolism.

The quantity of free nitrate or its reduction products in a particular tissue has been taken as evidence of assimilation, e.g. Eisenmenger, 1933, but it is clear that either a high level of nitrate or a reduction product in a tissue may indicate that it is normally metabolised there; or conversely that it is accumulating because of the absence of the assimilation system in that region. Nightingale et al., 1928, demonstrated the rapid appearance of nitrite and ammonia in roots of Narcissus following the application of nitrate, clearly showing that some reduction of nitrate can occur in the intact root. This gives little indication however, of the contribution of the root to the total nitrate reduction by the plant. Hewitt et al., 1954, and Spencer and Wood, 1954, found an accumulation of nitrate in shoots of cauliflower and tomatoes respectively, under conditions of molybdenum deficiency. Addition of molybdenum to the culture solution resulted in the disappearance within three hours, of nitrate and corresponding formation of reduction products, suggesting that nitrate reduction following application of molybdenum could occur in relatively intact though doubtless deficiency damaged shoots. Several

workers have reported the apparent absence of nitrate from the leaves and stems of deciduous trees (e.g. Nightingale, 1935, Peach; Thomas, 1927, apple.) indicating that nitrate reduction in these plants probably occurs mainly in the roots. It would be of interest to repeat this work using modern methods of nitrate estimation. This is apparently not a general phenomenon, however, since Dittrich, 1930, found considerable quantities of nitrate in the leaves of Sambucus and Carpinus.

Eckerson, 1924, suggested that the ability of homogenates or expressed juice from a particular plant organ to reduce nitrate might be taken as a measure of nitrate reducing capacity. The experience of Silver et al., 1954, with nitrate reductase in Neurospora and of Spencer, 1958, with nitrate reductase in excised wheat embryos, both of whom found reductase activity in homogenates, although the intact organism was unable to carry out reduction, would seem to argue against the use of this criterion.

It is probable that the use of $N^{15}O_3^-$ would lead to more precise information on the assimilation of nitrate by different tissues and organs of intact plants.

(2) Site in the Cell.

Clark and Shive, 1934, found that the rate of assimilation of nitrate by tomato roots was depen-

dent upon the pH of the ambient solution, and argued that since this was so the assimilating site must be near to the surface of the cells. Burstrom, 1937, was unable to find a similar influence of pH on nitrate assimilation by wheat roots. The limitation of this type of argument is most clearly emphasised, however, by recent work on the passive uptake of solutes by cells leading to the "apparent free space" concept, which suggests that the surface of the cytoplasm may not represent any appreciable barrier to the free diffusion of solutes to sites deeper in the cell.

Histochemical techniques have found little application in the locating of nitrate reducing systems within the cell. Inference as to location of the enzymes of nitrate assimilation must therefore be made from studies on cell fragments, and here there is a considerable gap in the published experimental work; interest having so far centred on the isolation and purification (i.e. concentrating the activity) of nitrate reducing preparations with consequent lack of attention to preservation of the cell fine-structure. It is of interest to examine briefly the ways in which cells have been fragmented prior to the estimation of nitrate reductase activity. Kastle and Elvolve, 1904, showed that the expressed juice of potato tubers was able

to convert nitrate to nitrite. Aldehydes were found to be particularly good "accelerators" of the process. Subsequently many other workers have used expressed sap for determinations of nitrate reducing activity. Presumably such preparations consist of a colloidal suspension of cell organelles in varying states of fragmentation and give little or no precise information on enzyme localisation.

Evans and Nason, 1953, extrated a nitrate reductase from leaves of Glycine max by grinding with alumina powder in a Waring blendor, followed by a Ten Brook homogenizer. After centrifuging at 20,000 g for ten minutes 10% of the nitrate reducing activity of this homogenate remained in solution in the supernatant. The 90% of sedimented reductase activity was presumably either bound onto insoluble cell particles or adsorbed onto the alumina powder. It was found that illuminated grana prepared by extracting leaves in cold 0.05M K_2HPO_4 buffer at pH 7.0 + 0.5% KCl, could "activate" this purified soluble nitrate reductase by acting as a source of TPNH. Thus it was inferred that nitrate reductase is normally situated in the cell near to the source of reduced nucleotide formation, but although the suggestion was made as early as 1924 by Ullrich, there is as yet no report of nitrate reduction or assimilation by isolated whole chloroplasts or even by any other well defined population

of cell-free particles.

(3) The Enzymology of nitrate reduction and assimilation.

Arising from the question of whether nitrate assimilation occurs in several organs of the plant and in various parts of the cell is the problem of whether the same enzyme systems are involved in all organs of the plant or all parts of the cell, or even at all stages during the life cycle of the plant. Suggestions that more than one enzyme may be involved in nitrate reduction in higher plants are inherent in several studies, but before examining this problem it is convenient to survey the properties of such enzymes as have been described.

NITRATE REDUCTASE:

Wu and Loo, 1950, reported the isolation of a nitrate reductase from soy bean sprouts which used lactate as an electron donor, but further characterisation of this enzyme does not appear to have been given. The nitrate reductase isolated from soy bean leaves by Nason and Evans was purified 67-fold by successive adsorptions on calcium phosphate gels and salting out with ammonium sulphate solutions. Inhibition of the enzyme by p-chloromercuribenzoate was reversed by cysteine hydrochloride, suggesting SH groups to be necessary for enzyme activity. Inhibition by cyanide, azide and thiourea indicated a metal requirement for the enzyme. TPNH and DPNH were

almost equally effective as electron donors and the stoichiometry of the reaction was found to follow the equation:-

$$NO_3^- + \underset{\text{or}}{\underset{\text{DPNH}}{\text{TPNH}}} + H^+ \rightarrow NO_2^- + TPN^+ + H_2O$$

The enzyme exhibited a well defined pH optimum at 6.0, the K_m with respect to nitrate, was 7.5×10^{-3}M and F.A.D. but not F.M.N. was found to enhance the activity of the enzyme. The purification procedure also resulted in an increased TPNH-cytochrome C reductase activity in the preparation; a result of considerable interest in view of the previously mentioned evidence of Kinsky et al., 1958, for the identity of the two enzymes of Neurospora. Nason and Evans concluded that since the nitrate reductase and TPNH-cytochrome C reductase activities were not parallel throughout the purification, the two enzymes must be distinct. This is not necessarily true. For instance, if two or more nitrate reductases were present in the crude extract, one of which was identical or closely associated with TPNH-cytochrome C reductase, a gradual elimination of this component could account for the non parallel activities. Evans and Hall, 1953, and 1955, using radioactive Mo^{99}, showed this metal to be associated with the enzyme and essential for its activity. This finding is in good agreement with the physiological studies of Hewitt et al., and Spencer et al., where a high concentration of nitrate in the

shoots of molybdenum deficient plants was found to disappear within a few hours of feeding molybdenum. It is of interest to note that in the work of Evans and Hall, ferrous and ferric ions brought about some reactivation of nitrate reductase preparations previously dialysed against cyanide containing buffer. (32% of the reactivation brought about by molybdenum). The animal enzymes, xanthine oxidase and aldehyde oxidase are both molybdoflavo proteins which also contain iron as a component of the prosthetic group, Reichert et al, 1954, Mahler et al., 1954. Both of these enzymes are capable of catalysing the reduction of cytochrome C, and the latter can also use nitrate as an electron acceptor. In view of these facts, and the possible association of cytochrome C reducing activity with nitrate reductase from higher plants, a careful investigation of iron metabolism in relation to the functioning of nitrate reductase in vitro would seem to be warranted.

Although nitrate reductase is known to be adaptively formed in many fungi and micro-organisms, the situation amongst higher plants is less clear. Nason and Evans, 1953, found normal activity in seedlings of soybean receiving only ammonium nitrogen, but free nitrate was found to be present in the leaves. Candella et al., 1957, found a lower, though still appreciable, nitrate reductase activity in leaf extracts of cauliflower plants receiving ammonium nitrogen than in plants receiving nitrate or nitrite nitrogen. These authors further showed that nitrate reductase

activity was high in mature leaves, but was much lower in senescent, and young rapidly expanding leaves. Subjection of plants to prolonged dark or anaerobic conditions also resulted in a reduced nitrate reductase activity. Spencer reports that excised wheat embryos grown in sterile culture with reduced forms of nitrogen show no nitrate reductase activity. Within 24 hours of adding nitrate, nitrate reductase is found in embryo homogenates, intact embryos, however, remain unable to use nitrate as a source of nitrogen. It is not yet clear whether this failure of embryos containing active nitrate reductase to utilise added nitrate is due to some in vivo difference in the enzyme, or to some other internal factor, such as a limiting supply of a suitable electron donor. A feature of this adaptively formed reductase in embryos is a specific requirement for DPNH as electron donor, whereas the nitrate reductase of mature leaves is able to use DPNH or TPNH as electron donor. The work of Hagemann and Arnon, 1955, with the enzyme glyceraldehyde phosphate dehydrogenase (G.D.P.) affords an interesting parallel with this situation. The G.D.P. enzyme from seeds and seedlings of garden pea germinated in the dark was DPNH specific, but in the shoots of seedlings germinated in the light there appeared, after the tenth day, a TPNH linked enzyme. Glyceraldehyde phosphate dehydrogenase from the roots remained DPNH specific throughout the life cycle of the plant. Of further interest in this connection is the data of Mason and Evans, 1953, suggesting

DPNH to be superior to TPNH as an electron donor for nitrate reductase from the roots of tomato. It is a tempting generalisation that the photosynthetic system is the main source of reduced TPN in higher plants, also that the TPNH linked enzymes are located mainly in the photosynthetic tissues and are possibly adaptively formed in response to light. In the dark, and in non-photosynthesising regions on the other hand, DPNH is found to be the dominant electron donor.

NITRITE REDUCTASE:

Evans and Nason, 1953, found that crude extracts of soy bean leaves and tomato roots which they were assaying for nitrate reductase activity were able to catalyse a slow decomposition of nitrite. The products of the reaction were not identified and the reaction was inhibited by 10^{-3}M hydroxylamine hydrochloride. Nason et al., 1954, obtained a partial purification of soy bean leaf extract catalysing the reduction of nitrite and hydroxylamine to ammonia. TPNH or DPNH functioned as electron donors, and the system was stimulated by the addition of FMN or FAD and manganese. These preliminary results suggest similarities with the nitrite reductase from Neurospora, but further detailed work is needed before such a generalisation is justified. At the physiological level, study of the metabolism of nitrite is rendered difficult because of its toxicity

at relatively low concentrations. Despite this difficulty, however, there are several reports of limited growth and protein synthesis by plants in water culture, using nitrite as a source of nitrogen. Anderson, 1924, Dittrich, 1930, and subsequently many other workers have reported the occurrence of nitrite in plant tissues, although always in small quantities. Steinberg, 1939, emphasised the non-specificity of current tests for nitrite, nitrohydroxylaminic acid, in particular, giving the same colour reactions. But allowing for this source of uncertainty, there would seem to be general agreement that nitrite is formed in tissues as a result of nitrate reduction. The reason for the consistently low levels of nitrite observed in plant tissues is not understood; a high turn-over number for the nitrite reducing enzymes, whilst an explanation, has not as yet been demonstrated.

HYPONITRITE REDUCTASE:

No hyponitrite reductase of the type described from Neurospora has yet been found in higher plants. Frear, 1956, and Burrell and Frear, 1958, infiltrated leaf sections of soy bean with 0.01M solutions of sodium hyponitrite labelled with N^{15}, and found, after one hour, N^{15} present in ammonia and amide in the tissue. Sodium hyponitrite is however, an unstable compound, and it is as yet uncertain whether its decomposition prior to the formation of ammonia and amide was in fact an enzyme

mediated reaction. Illumination of the leaf sections during infiltration did not affect the amount of hyponitrite reduced, whether or not sections treated in this way were able to carry out photosynthesis was not investigated.

HYDROXYLAMINE REDUCTASE:

Wood, 1953, after examination of available evidence concluded that "it is doubtful whether the occurrence of free hydroxylamine has ever been demonstrated in plant tissues", a conclusion which would still appear to be valid. The same author was, however, 1948, able to obtain some growth and protein synthesis in oat plants grown under approximately sterile conditions in water culture, using 10^{-5}M hydroxylamine as the sole source of nitrogen. Yemm and Willis, 1956, also observed a limited assimilation of hydroxylamine in excised barleyroots. There is as yet, however, no detailed description of hydroxylamine reducing enzyme(s) from higher plant tissues.

In connection with the possible occurrence of hydroxylamine in plant tissues, certain indirect and entirely speculative evidence is of some interest. Pirson et al., 1952, and Kessler, 1955 & 1957, showed that manganese deficiency in green cells has an effect on photosynthesis, and in particular, upon the oxygen evolving rather than the photoreducing system. Other functions, including respiration and chlorophyll forma-

tion appeared to be relatively unaffected by the deficiency. Hydroxylamine resembled manganese in apparently inhibiting only the oxygen evolving system of photosynthesis. As previously discussed, Nicholas, 1957, showed manganese to be a component of the hydroxylamine reductase of Neurospora. It therefore seems possible that if a similar reductase were located in the chloroplast, conditions of manganese deficiency would result in an impeded hydroxylamine reductase activity and consequent piling up of hydroxylamine to inhibitory concentrations. In this way Kessler's observation of the similarity of hydroxylamine inhibition and manganese deficiency would be explicable as a single effect. Extending this idea, the hydroxylamine accumulation would eventually inhibit the photolysis of water and hence TPNH formation. Such a reduced formation of electron donor would, in turn, limit the extent of nitrate reduction, affording a possible explanation of the high levels of nitrate found in leaves of manganese deficient plants, Leeper, 1941, Hewitt et al., 1949.

(4) The Problem of Intermediates.

Considering a generalised metabolic sequence $A \longrightarrow B \longrightarrow C \longrightarrow D \longrightarrow E$ in which B, C and D are toxic to the cell at sites other than those at which they are synthesised or broken down. Originating probably from the work of Mothes, 1938, was the suggestion that B, C and D might be trans-

formed as rapidly as they are formed thus avoiding affecting the rest of the cell. As Burstrom, 1945, pointed out, however, it is questionable whether such a rapidity of reaction could avoid the possibility of a gradual leakage of B, C or D to vulnerable sites. It is therefore of interest to consider possible mechanisms for constraining the movement of B, C and D within the cell. This Burstrom did in a "plasma colloid" hypothesis, suggesting the toxic intermediates of nitrate reduction to be chemically bound to some component of the cytoplasm whilst undergoing reduction. Identification of such complexes would undoubtedly present considerable analytical difficulties but a possible approach to this problem lies in the work of Rittenberg and Krasna, 1955, who suggested examining the properties of synthetic chemical models of the reaction sequence. of also the work of Winfield, 1956, on ruthenium complexes as models for the action of catalase. Such a reaction sequence involving possibly protein - metal - intermediate complexes implies an ordered arrangement of molecules in a sort of crystal in the cell fine structure; a structure which might ultimately be analysed by electron microscope techniques.

(5) Respiration in relation to nitrate assimilation.

Technical difficulties, mainly associated with the existence of a diffusion pathway, have restricted the study of this aspect of respiration in intact higher plants. Using isolated leaves of Ligustrum Ruhland and Ulrich, 1929, and tomato, Hanner, 1935, observed a stimulation of respiration and rise in R.Q. to a value greater than 1.0 following feeding of nitrate. Postma, 1939, found excised roots low in carbohydrate unable to assimilate nitrate. It may be, of course, that the carbohydrate starvation had in fact affected the nitrate reducing system. Application of nitrate to such tissues did however, result in an increased rate of respiration. This result was explained in terms of anion respiration associated with the uptake of nitrate ions into the root cells. Hoagland, 1944, Folkes et al., 1952, and Willis et al., 1955, measured the carbondioxide output by whole seedlings and isolated organs of germinating barley, following addition of nitrate or ammonia. Data from isolated roots suggested that a large proportion of the increased carbon dioxide production following addition of nitrate or ammonia, came from these organs. This work respresents probably the closest attempt so far to estimate the contribution of a particular organ to the total nitrate reduction

carried out by the plant. It is not useful here to collect the widely scattered data on nitrate reduction in relation to respiration. Certain points, however, may be made: Plants at different stages of development appear to differ in certain aspects of nitrate reduction; thus in seeds germinating in the dark there is a DPNH-linked nitrate reductase apparently adaptively formed in response to the presence of nitrate. The coupling of nitrate reduction to respiration might be through the reduced nucleotide generated in the course of respiration. In the case of wheat there is known to be an ontogenetic production of a TPNH-nitrate reductase. Whether this is coupled to reduced nucleotide formed in the course of respiration or photosynthesis is not as yet clear. (Spencer, unpublished data). In roots it would seem possible that the proportion of nitrate reduction which does occur, (and the amount probably varies considerably between species), is linked to reduced nucleotides, e.g. DPNH generated in the course of respiration. c.f. Mason & Evans, 1953, data on tomato roots.

In green tissues a coupling of nitrate reduction to TPNH of photosynthetic origin probably predominates over a respiration-linked system. Such a generalisation must be examined in relation to what is known of the effect of light on nitrate reduction

in higher plants.

(6) Light in relation to nitrate assimilation.

The coupling of nitrate reduction to TPNH generated in the course of photosynthesis has been discussed in connection with the properties of nitrate reductase isolated from green leaves. It remains to be shown whether such a system can account for the observed facts concerning the interaction of light and nitrate in intact plants.

A direct approach to this problem was made by Stoy, 1955, who compared the action spectra of nitrate assimilation and carbon dioxide assimilation in isolated wheat leaves. The action spectra were found to be very similar in white and red light, but nitrate reduction was promoted at shorter wavelengths. Stoy, 1955, speculated that the absorption at short wavelenghts might be due to a yellow pigment of the riboflavine type. A light-induced reduction of the vitamin might be reversed by nitrate acting as an electron acceptor. It is of interest to note that dehydroascorbic acid can also serve in the oxidation of reduced riboflavin, Brauner and Brauner, 1954. A possible electron transfer might therefore take place from riboflavin to ascorbic acid to nitrate.

Virtanen and Saubert von Hausen, 1949, grew wheat embryos from which the endosperm had been

removed, after soaking the grain for one hour in tap water, in sterile culture solutions containing nitrate or ammonium as the source of nitrogen. It was found that seedlings grew on nitrate, only if ascorbic acid was also added to the culture solution. Plants receiving ammonium nitrogen grew well without added ascorbic acid. The ascorbic acid proved to be replaceable by other reducing compounds such as reduced glutathione, reductone and cysteine. The nature of this interaction of nitrate and ascorbic acid does not appear to have been studied further. Chapter I of this thesis describes preliminary experiments intended to examine further the effect of ascorbic acid on the reduction of nitrate, using the tomato plant as the subject of experiment. As a preliminary to this work plants were grown in water cultures using either nitrate or ammonium ions as the source of nitrogen in order to establish growth standards for comparison, and to make measurements of the ascorbic acid content of the tissues. As will be seen later, considerable differences were observed between the growth of plants receiving nitrate and ammonium nitrogen and it is with a description of these differences and an investigation of certain of the metabolic differences underlying them that Chapters II and III are concerned. In the course of this work reference is made to the inform-

ation on nitrate and ammonia metabolism considered in the introduction. The problem of how ascorbic acid affects nitrate assimilation remains unanswered.

CHAPTER I.

ASCORBIC ACID IN RELATION TO NITRATE REDUCTION.

INTRODUCTION:

For the purpose of examining the effect of ascorbic acid on plant growth when nitrate or ammonia are the sources of nitrogen, it is first desirable to examine the growth of such plants under otherwise standard experimental conditions, before considering the effects of added vitamin C.

Unfortunately the facilities available for growing plants did not permit the degree of control of environmental conditions which would have been desirable. Plants were grown in water cultures in a greenhouse. The placing of rafters in the greenhouse resulted in an uneven illumination of the cultures; in order to avoid systematic differences due to this rafter shading, culture vessels were placed in a statistically randomised arrangement on the greenhouse benches, in all the experiments described. As an additional precaution, where all the plants from particular culture vessels were harvested at the same time these cultures were taken at random from the treatment concerned. The relative humidity in the greenhouse was recorded in

the course of most experiments, using a thermohygrometer; big variations in the relative humidity occured with the time of day and with the season. There was no control of humidity in the greenhouse. The greenhouse was equipped with a thermostatically controlled heating system capable of maintaining a minimum temperature of 56° F on the coldest nights; on hot days in summer the air temperature in the greenhouse rose to over 110° F, the opening of windows and introduction of shading from overhead blinds afforded some protection against these extreme conditions, but there was no air cooling system available in the greenhouse. In winter time, the light intensity in the greenhouse fell well below the optimum for plant growth, in some experiments the daylight was supplemented by 300 Watt lamps suspended above the water cultures. In view of the extreme environmental conditions experiments were not carried out in midsummer, (December and January), or midwinter (June and July), but despite this, marked differences in plant growth rates from one experiment to the next, due to the seasonal changes in environmental conditions, will be noticed in the data to be presented.

EXPERIMENT I. THE GROWTH OF YOUNG TOMATO PLANTS IN WATER CULTURES CONTAINING NITRATE AND AMMONIUM NITROGEN.

INTRODUCTION:

In the first experiment, the growth of young

tomato plants on three types of culture solution is compared.

(i) "Complete" culture solution containing both nitrate and ammonium nitrogen;

(ii) Culture solution containing only nitrate nitrogen;

(iii) Culture solution containing only ammonium nitrogen.

MATERIALS AND METHODS:

Plants.

Commercial seed of tomato var. Grosse Lisse was surface sterilised by:- wetting in 95% ethanol, rinsing in distilled water, soaking for half an hour in 1% formalin and then rinsing again in freshly distilled water. The seed was then placed on a damp filter paper in a petrie dish and placed in an incubator at 25° C. When the radicle was about half an inch in length, i.e. after approximately two days, the seedlings were transferred to waxed mosquito netting stretched over a rectangular glass frame which was then placed in a pyrex pie dish of the same shape, containing a continuously aerated, dilute, culture solution at pH 6.0. When the cotyledons were fully expanded, i.e. after about seven days, the now nine-day old seedlings were transferred to culture vessels consisting of 500 ml. beakers. Three plants were grown per beaker, each being held with a cotton wool plug, between the halves of a split cork, previously soaked in molten wax to prevent fungal growth.

The beakers were surrounded with bible paper, the black face innermost excluding light and thus reducing algal growth in the culture solution, and the white outer face reflecting light and minimising over-heating of the vessels.

2. Culture Solutions.

The composition of the culture solutions used is given in Table I, the complete culture solution is essentially that of Arnon and Hoagland, 1940, but at one-fifth of the concentration used by these authors. In culture solutions containing all the nitrogen in the form of nitrate, phosphate is supplied as KH_2PO_4. In all ammonium-nitrogen solutions potassium and calcium are added as the chlorides.

For each type of culture three initial pH's were used, approximately 4.2, 5.6 and 6.8; these were obtained by the addition of an appropriate amount of $\frac{N}{50}$ sodium hydroxide or $\frac{N}{50}$ hydrochloric acid, following titration of a sample of the culture solution at a glass electrode. Three replicate cultures were set up at each initial pH value.

RESULTS.

After placing the plants in the cultures the drift in pH of the culture solution was recorded periodically, Table II, figs. 1, 2, 3.

The acidity developed in ammonium cultures is

TABLE I. THE COMPOSITION OF CULTURE SOLUTIONS.

Culture Solution.	Macronutrients.		Micronutrients.	
Complete solution.	KNO_3	0.0020M	B	0.100ppm.
	$Ca(NO_3)_2$	0.0006M	Mn	0.100ppm.
	$NH_4H_2PO_4$	0.0004M	Zn	0.010ppm.
	$MgSO_4$	0.0004M	Cu	0.004ppm.
	Fe (EDTA)	0.6ppm.	Mo	0.004ppm.
All Nitrate solution.	KNO_3	0.0020M	Fe (EDTA complex)	0.6 ppm.
	$Ca(NO_3)_2$	0.0006M	B	0.100ppm.
	KH_2PO_4	0.0004M	Mn	0.100ppm.
	$MgSO_4$	0.0004M	Zn	0.010ppm.
	$NaNO_3$	0.0004M	Cu	0.004ppm.
			Mo	0.004ppm.
All Ammonium solution.	KCl	0.0020M	Fe (EDTA)	0.6 ppm.
	$CaCl_2$	0.0006M	B	0.100ppm.
	$NH_4H_2PO_4$	0.0004M	Mn	0.100ppm.
	$MgSO_4$	0.0004M	Zn	0.010ppm.
	NH_4Cl	0.0032M	Cu	0.004ppm.
			Mo	0.004ppm.

TABLE II. EFFECT OF GROWING TOMATO PLANTS, ON THE pH OF CULTURE SOLUTIONS CONTAINING DIFFERENT FORMS OF INORGANIC NITROGEN. ALL pH VALUES ARE THE MEAN FROM THREE REPLICATE CULTURES.

Culture Solution.	Initial pH.	Day 3. pH	Day 5. pH	Day 7. pH	Day 9. pH	Day 11. pH	Day 13. pH	Day 15. pH
Nitrate.	4.09	4.26	4.25	4.66	5.17	6.16	6.65	7.17
	5.43	5.86	5.98	6.23	6.37	6.69	6.84	7.34
	7.09	7.16	7.13	7.22	7.25	7.36	7.33	7.64
Complete.	4.06	4.03	4.08	4.25	4.54	5.20	5.40	5.82
	5.52	5.24	5.19	5.38	5.32	5.64	6.34	6.42
	6.99	6.96	6.88	6.86	6.69	6.70	6.61	7.31
Ammonia.	3.95	3.98	3.94	3.92	3.84	3.94	3.82	3.80
	5.43	4.66	4.24	4.07	3.75	3.86	3.73	3.76
	6.82	6.52	6.23	5.83	4.26	3.90	3.61	3.60

Figure 1 — Change in pH plotted against time for culture solutions of initial pH 4·0.

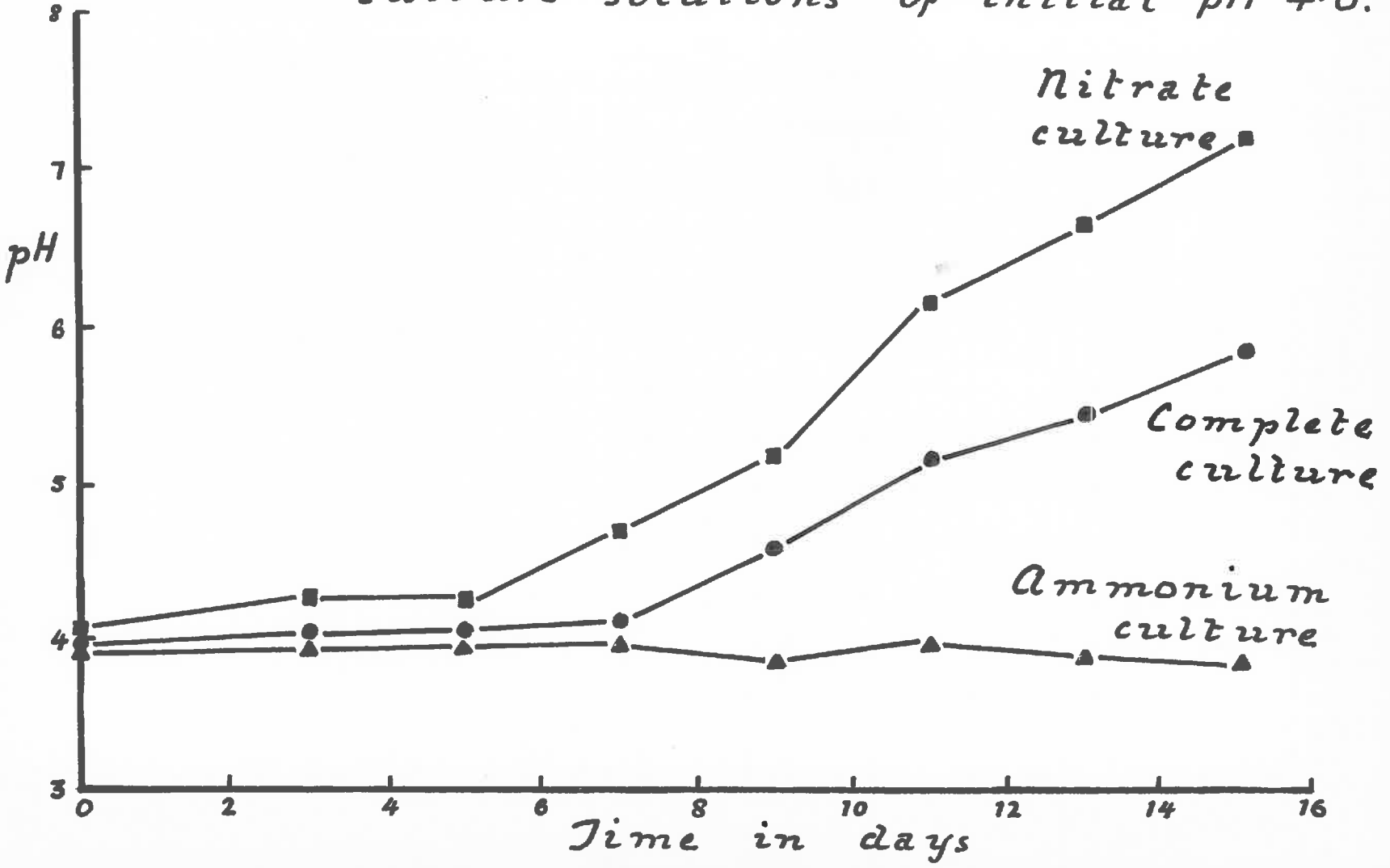


Figure 2 – Change in pH plotted against time for culture solutions at initial pH 5.5.

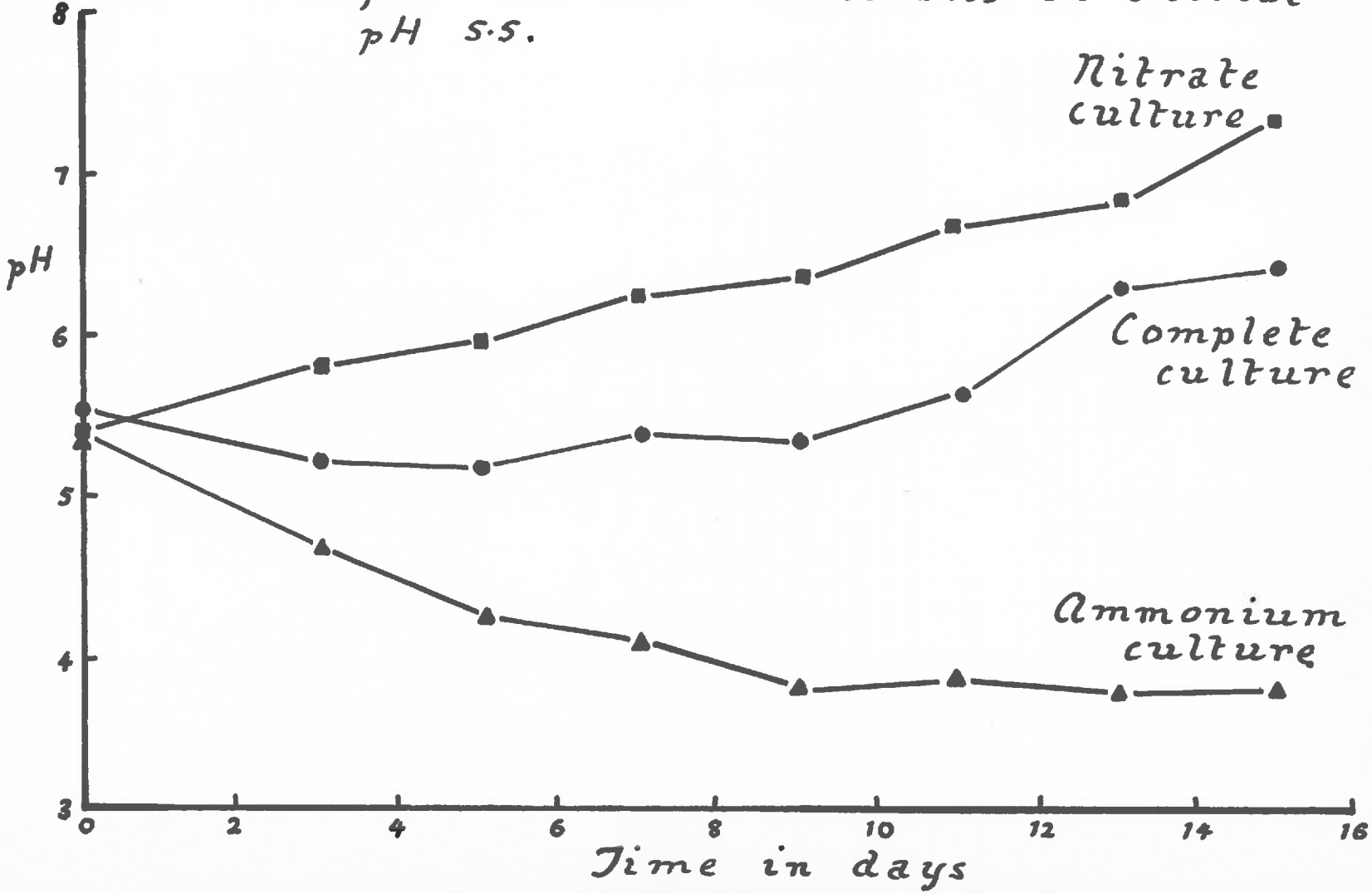
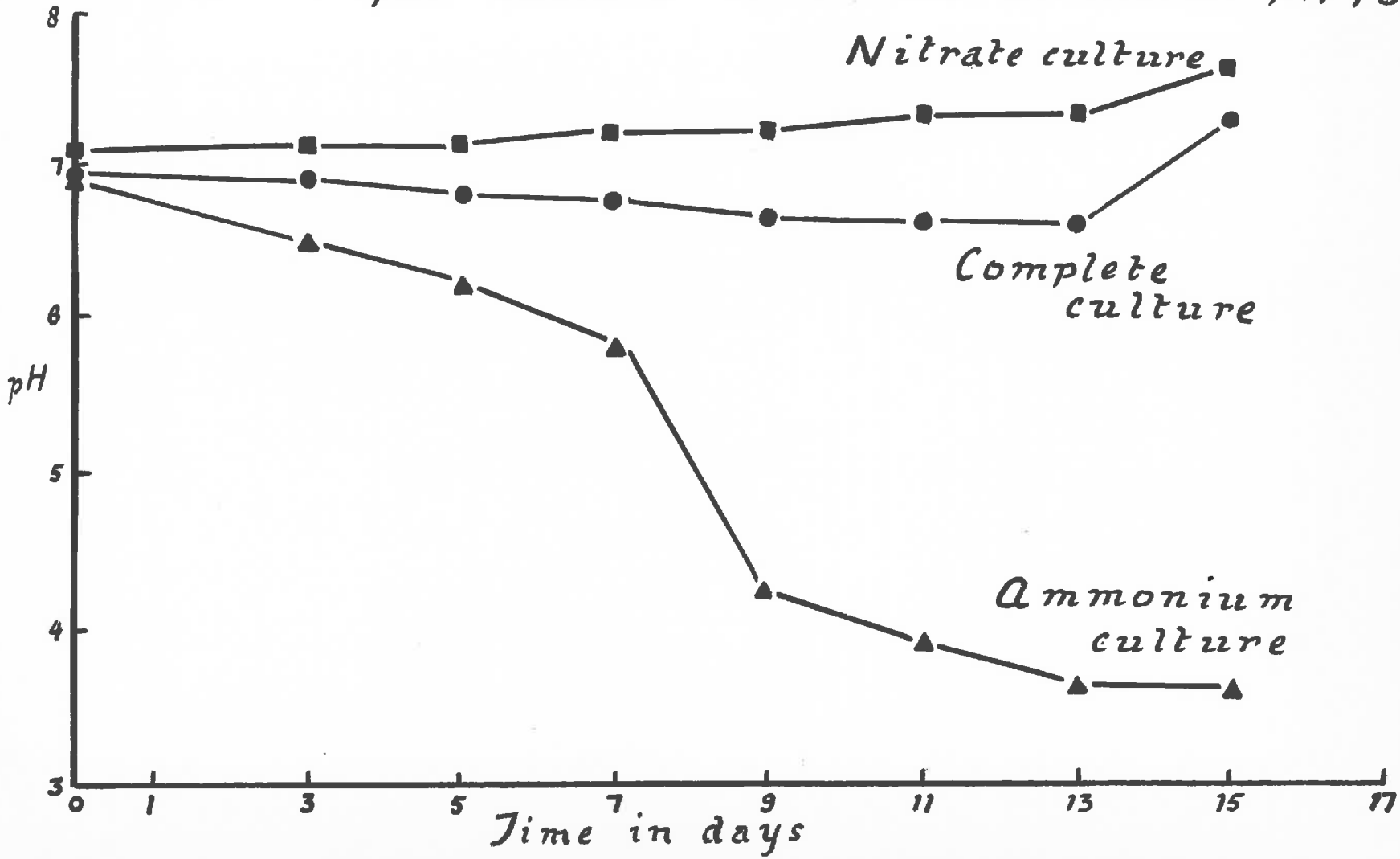


Figure 3 — Change in pH plotted against time for culture solutions at initial pH 7·0.



generally attributed to the excretion of hydrogen ions consequent on ammonium ion uptake. Hecking, 1956, applied the "Base excess" concept, Johnston, 1916, to continuously flowing culture solutions in which young maize plants were actively growing and calculated that under certain conditions of pH and low ammonium ion concentration there was a 1 : 1 ratio of ammonium ion uptake to hydrogen ion excretion. Ammonia loss to the atmosphere, bacterial contamination of the culture solution, and other uncontrolable factors in the culture technique adopted, make application of the "Base Excess" calculation impracticable in this experiment. The rise in pH in all-nitrate cultures, Table II, suggests an excretion of hydroxyl ions or concurrent uptake of hydrogen ions when nitrate is taken up by the root systems, there appears to be no detailed study of this effect however, in the literature. If in fact there was a 1 : 1 ratio of ammonium ion uptake to hydrogen ion excretion and of nitrate ion uptake to hydroxyl ion excretion, then the 8 : 1 ratio of NO_3^- : NH_4^+ in the "Complete" culture solution which does not change pH appreciably during the course of the experiment, would tend to suggest relative rates of uptake of this order i.e. ammonium ions taken up at eight times the rate of nitrate ions. The small but consistent lowering of pH, in "Complete" cultures, followed by a subsequent rise in pH could be explained by an initial more rapid uptake of ammonium than of nitrate ions resulting in an

initial excess of hydrogen ions. At about the thirteenth day the ammonia in the culture becomes exhausted and the pH rises again. The absence of this initial fall in pH in "Complete" cultures at a starting pH of 4.0 is perhaps due to the "damping" effect of the logarithmic scale at the higher hydrogen ion concentration. This discussion presumes, without good reason, that the buffering of the culture solution following differential uptake of other inorganic ions, or excretion of organic molecules by the roots, does not vary with the form of the inorganic nitrogen supply.

In relation to this question, it must also be noted that there is no a priori reason for supposing that the metabolic activities of the plant, which might result in effects on the pH of the culture solution in which it is growing is standard, they may well change with the physiological age of the plant.

Table III shows the effect of the ammonia culture in giving plants of lower fresh and dry weights of both roots and shoots. Plants grown on ammonia cultures at initial pH 6.82 produced significantly higher fresh and dry weight yields than plants in similar cultures at initial pH's 3.95 and 5.43. The ultimately lower pH of ammonia cultures at 5.43, Table II, probably result from a greater hydrogen ion secretion by the larger (dry weight 24 mgms.) plants at initial pH 6.8 than by the smaller (dry weight 7 mgms.) plants in

TABLE III. FRESH AND DRY WEIGHTS OF PLANTS GROWN IN CULTURE SOLUTIONS CONTAINING AMMONIUM AND NITRATE IONS. PLANTS HARVESTED 15 DAYS AFTER PLACING IN THE CULTURES. ALL VALUES ARE THE MEANS OF TWELVE PLANT WEIGHTS.

Culture Solution.	Initial pH.	Shoot Weight gms.		Root Weight gms.	
		Fresh Weight.	Dry Weight.	Fresh Weight.	Dry Weight.
Nitrate.	4.09	0.762	0.0468	0.150	0.0074
	5.43	0.788	0.0493	0.146	0.0072
	7.09	0.758	0.0477	0.167	0.0083
Complete.	4.06	0.722	0.0442	0.178	0.0080
	5.52	1.007	0.0632	0.213	0.0097
	6.99	1.204	0.0762	0.263	0.0126
Ammonia.	3.95	0.061	0.0056	0.014	0.0014
	5.43	0.110	0.0109	0.018	0.0014
	6.82	0.235	0.0211	0.037	0.0035

cultures having initial pH's of 3.95 and 5.43. The differences in dry and fresh weights between plants grown in complete and in all nitrate cultures are not statistically significant. Plants grown in complete cultures solutions at initial pH 4.03 tend to be smaller than plants in similar cultures at initial pH's 5.52 and 6.99, a result which though barely significant statistically, suggests a deleterious effect of the low pH in the first ten days, i.e. before the pH rises to a value of about 5.0 comparable with that of the other cultures. Plant growth as measured by leaf, shoot and root lengths varied according to composition and initial pH of the culture solution in the same general direction as fresh and dry weights. (Table IV).

It is of interest to note that the cotyledons continued elongating throughout the period of the experiment, a process also influenced by the conditions in the nutrient solution.

The data obtained from this experiment suggests that the growth of young tomato plants is made less vigorous by culture solution pH's of below approximately 5; however, it is not established that this is an effect of pH per se or rather an effect of the high hydrogen ion concentration on the uptake of other ions. Of particular interest would be to know whether the poor growth of tomato plants at pH 4 is due to the inability of the plants to exclude hydrogen ions from vulnerable

TABLE IV. LEAF, SHOOT AND ROOT LENGTHS IN CMS. OF TOMATO PLANTS GROWN IN CULTURE SOLUTIONS CONTAINING NITRATE AND AMMONIUM IONS. EACH VALUE IS THE MEAN OF 12 LEAF-MEASUREMENTS.

Culture Solution.	Initial pH.	Length of Cotyledons. Day 8.	Day 10.	Day 15.	Length of 1st Leaf. Day 8.	Day 10.	Day 15.
Nitrate.	4.05	2.2	2.6	4.1	1.7	2.9	7.0
	5.43	2.3	2.6	4.0	1.8	3.0	7.1
	7.09	2.0	2.5	3.9	1.6	2.6	6.6
Complete.	4.06	1.9	2.3	3.8	1.6	2.8	6.7
	5.52	2.3	2.7	4.3	2.0	3.2	7.7
	6.99	2.5	2.8	4.5	2.2	3.5	7.7
Ammonia.	3.95	1.2	1.4	1.5	0.8	0.9	1.3
	5.43	2.0	2.1	2.6	1.2	1.9	2.1
	6.82	2.0	2.3	2.9	1.4	1.6	3.1

TABLE IV. (continued)

Culture Solution.	Length of 2nd Leaf. Day 8.	Day 10.	Day 15.	Length of 3rd leaf Day 15.	Length of 4th. leaf Day 15.	Length shoot apex to root apex Day 8.	Day 15
Nitrate.	0.7	1.9	6.5	2.4	0.9	11.6	27.0
	0.7	1.8	6.8	2.8	1.0	12.0	26.7
	0.7	1.8	6.4	2.6	1.0	11.3	27.3
Complete.	0.6	1.6	6.4	2.9	1.1	9.4	24.9
	0.9	2.2	7.2	3.3	1.4	11.2	26.8
	1.2	2.8	7.5	4.2	1.7	12.3	28.8
Ammonia.	-	-	0.6	-	-	6.3	8.6
	0.7	1.2	1.6	-	-	7.7	9.8
	0.7	1.1	2.4	-	-	10.6	13.6

sites within the cell, against a concentration gradient; if this were so then it is probable that acid tolerant species do possess such a mechanism. Alternatively, it might be supposed that the hydrogen ions are ably to permeate the cell, in which case the cell constituents of the acid tolerant species presumably have lower pH inactivation values. Haas, 1920, added lime to soils of different acidities and reported changes in the pH of the sap of plants growing in these soils. Newton, 1923, found that under conditions of limiting calcium supply the sap pH was not affected; however, as Small, 1955, points out, liming the soil may affect many aspects of plant growth in addition to the pH of the expressed juices, e.g. the question of iron availability, and effects on the cell walls, so that studies of this type do not give much information on the effect of external hydrogen ion concentration on internal pH. Broyer, 1951, states that "In living tissues, a tendency toward modification of the hydrogen ion concentration by a moderate alteration of the pH of the external medium is counteracted within the protoplasm by a direct buffer action of constituent compounds and, possibly, by a similarly effective modification in the rates of some metabolic reactions." There would appear to be no good evidence for this statement, Broyer presented as supporting evidence, measurements of the pH of sap exuding from cut stumps of tomato plants growing with their roots

in culture solutions of varying pH. There would seem no necessity to suppose that the pH of such an exudate should reflect the pH prevailing within individual cells of the root. Bonner, 1934, studied the effects of hydrogen ion concentration on cell elongation and suggested that it might be due to swelling of the pectic matrix of the cell wall due to ionisation of carboxyl groups, an extension of Bonner's work could lead to a further understanding of the primary effects of hydrogen ions on plant cells.

From the results of this experiment it is clear that the pH of ammonium cultures must be maintained at approximately pH 6, in order to obtain tomato plant growth comparable with that obtained in nitrate or complete cultures.

It is perhaps pertinent at this point to enquire whether the culture technique adopted is that most suitable for the type of experiments to be carried out. It is possible, for example, that a continuously flowing culture would be more suitable; convenience of manipulation is the main reason why in experiments carried out so far, the technique described above has been retained.

EXPERIMENT II.

The control of pH in ammonium cultures.

INTRODUCTION.

Maré, 1914, and subsequently many other workers

endeavoured to control the pH of ammonium cultures by addition of calcium carbonate. For two main reasons this procedure was not adopted; firstly, under the local greenhouse conditions, the presence of almost any source of carbon in a culture solution resulted in an excessive growth of bacteria and hence of course, serious modification of the composition of the culture solution: secondly, it is considered possible that the insoluble carbonate might preferentially adsorb components of the culture solution rendering them relatively unavailable to the plant. The pH of the culture solution was therefore maintained above pH 6.0, by periodic addition of appropriate quantities of $\frac{N}{50}$ sodium hydroxide solution.

MATERIALS AND METHODS.

Seedlings were raised and placed in culture solutions in the manner described in Experiment I. Complete, nitrate, and ammonium cultures were prepared as in Experiment I and four replicates of each culture type were set up. The pH of the culture solutions was maintained above pH 6.0, by addition of an appropriate quantity of $\frac{N}{50}$ sodium hydroxide following titration of a sample of the culture solution at a glass electrode. After 15 days one plant was removed from each culture vessel and the leaf lengths and fresh weights recorded.

The remaining two plants from each culture were harvested after twenty days and similarly measured.

RESULTS.

Table V and Figure 4 show the amounts of $\frac{N}{50}$ sodium hydroxide added to the cultures at various stages of the experiment up to the twelfth day.

From Table VI it is seen that, as in Experiment I, the growth of plants in ammonium cultures is less than in complete, or nitrate cultures. The difference between treatments however, is not apparent until after the fifteenth day and does not seem to affect the growth of the cotyledons, as in Experiment I. From the data presented, this improvement in growth of plants in ammonia culture would be attributable to the controlling of pH. At the time of the second experiment there was, however, a seasonal lowering of the light intensity in the greenhouse which limited the growth of all plants and in particular those receiving nitrate, so that a close comparison of the two experiments is not possible. The possible relationship between light and nitrate assimilation was reviewed earlier and is studied further in Chapter III.

From Table V and Figure 4 it is seen that the amount of sodium hydroxide added to an ammonium culture increases steadily with time. The amount of

TABLE V. Mls. $\frac{N}{50}$ NaOH added to nitrate, ammonium and complete cultures in order to maintain the pH at 6.5 approximately. All values are the mean amount of acid or alkali added to four cultures.

Culture.		2 days.	4 days.	6 days.	8 days.	9 days.	12 days.
Nitrate.	pH	6.55	6.70	6.61	6.95	6.75	6.84
	ml. $\frac{N}{50}$ HCl added.	-	-	0.9	0.9	-	-
Complete.	pH	6.28	5.54	6.11	6.50	6.51	6.69
	ml. $\frac{N}{50}$ NaOH added.	-	6.9	3.4	1.3	1.0	-
Ammonia.	pH	5.50	6.15	5.50	5.83	4.66	4.51
	ml. $\frac{N}{50}$ NaOH added.	4.6	4.4	6.9	4.7	12.1	12.9

Figure 4 — Total N/50 NaOH in ml. added to each culture plotted against time in days.

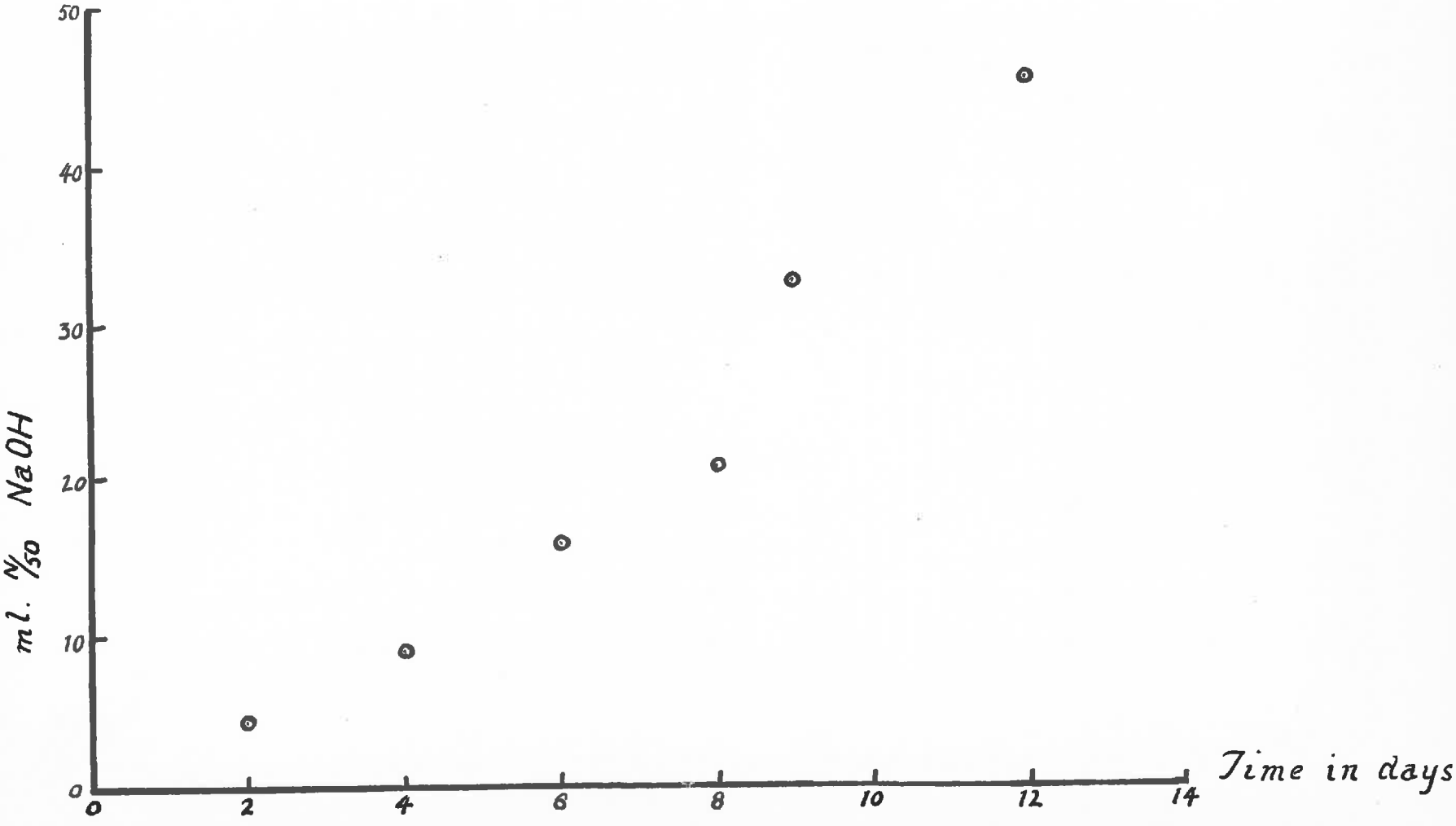


TABLE VI. LEAF LENGTHS AND FRESH WEIGHTS OF PLANTS HARVESTED AFTER 15 AND 20 DAYS. THE VALUES AT FIFTEEN DAYS ARE THE MEAN FRESH WEIGHTS AND LEAF LENGTHS OF FOUR PLANTS. THE VALUES AT TWENTY DAYS ARE THE MEAN FRESH WEIGHTS AND LEAF LENGTHS OF EIGHT PLANTS.

Culture Solution.	Time in Days.	Length of Cotyledons.	Length of 1st leaf.	Length of 2nd leaf.	Length of 3rd leaf.	Length of 4th leaf.	Length from shoot apex to root apex.	Fresh weight gms.
Nitrate.	15	4.2	4.3	3.0	0.4	-	20.8	0.415
	20	4.3	8.4	8.8	5.1	2.8	24.4	1.734
Complete.	15	4.4	4.9	3.6	0.8	-	18.1	0.432
	20	4.9	9.1	9.4	5.5	3.2	30.6	1.988
Ammonia.	15	4.3	4.1	3.4	0.5	-	16.2	0.419
	20	4.8	6.9	6.5	3.2	1.2	19.2	0.924

alkali to be added at any particular time probably depends on many factors, e.g.:-

(i) The amount of $\frac{N}{50}$ sodium hydroxide added to a culture is determined by titration of a sample of the culture solution at a glass electrode. The titre will depend on the chemical buffering of the culture solution which will be different over different parts of the pH range and which will also change with time as ions are taken up from the solution by the roots.

(ii) As will be shown later, ammonia gas is lost from an aerated culture solution, the rate of loss varying with pH of the solution. This loss will also affect the sodium hydroxide titration value. This loss of ammonia from the culture solution is greater from alkaline than from acid solution, so the possibility exists that if the amount of ammonia originally supplied (0.0036M.) represented a toxic concentration then the beneficial effect of the addition of sodium hydroxide may be due, in some measure, to its effect in reducing the ammonia concentration. See also Chapter II.

EXPERIMENT III. THE EFFECT OF NITRATE VERSUS AMMONIUM CULTURES ON THE GROWTH AND ASCORBIC ACID CONTENT OF YOUNG TOMATO PLANTS.

INTRODUCTION:

Despite the previously recorded, modified growth of plants when the nitrogen supply is in the ammonium form, it was decided to examine the effect of the form of the inorganic nitrogen supply on the ascorbic acid content of the plants.

MATERIALS AND METHODS:

(1) The production of uniform populations of tomato plants: Seeds of tomato var. Grosse Lisse were germinated under the conditions previously described, but in an attempt to improve the uniformity of the seedling population certain selections were carried out. The first selection was made on time of emergence of the radicle, all seeds from which the radicle protruded within a given twenty-four hour period, being regarded as belonging to a population. At 25° C., plants germinating between 48 and 60 hours provided a relatively uniform population although there was an indication that a twelve-hour selection period would have yielded further improvement in uniformity. From observation, but without measurement, it appeared likely that an initial selection based on seed size would have given

greater uniformity to the population. At the time of selection on radicle-emergence, the seedlings were transferred to a supporting tray placed over a gently aerated, dilute, culture solution, as previously described. The final selection, based on general uniformity of plant size, was made at the time of transfer of the seedlings to the culture vessels, i.e. when the cotyledons are expanded.

(2) Culture vessels and solutions.

In order to reduce the entanglement of roots and to make less frequent the need for correcting of pH, two litre beakers were used as culture vessels instead of the half-litre beakers as in earlier experiments. Masonite lids were used for the vessels, each taking four plants held by cotton wool plugs between the halves of a split cork. Lids and corks were soaked in molten wax prior to use in order to reduce fungal growth. The arrangement of the cultures was otherwise as previously described. The composition of the culture solutions was the same as that used in Experiment I (Table I), except that sodium nitrate was omitted from the all-nitrate solution. At the commencement of the experiment all culture solutions were adjusted to pH 6.8 by addition of an appropriate quantity of $\frac{N}{50}$ sodium hydroxide. The pH of the culture solutions was recorded daily, and corrected to within the limits 6.4 - 6.9 by

the addition of appropriate quantities of $\frac{N}{50}$ sodium hydroxide or $\frac{N}{50}$ hydrochloric acid.

(3) Experimental design and harvesting.

Six cultures (24 plants) of each treatment, i.e. complete, all ammonia nitrogen, and all nitrate nitrogen, were grown, and one plant removed from each culture at four intervals, 14, 17, 20 and 22 days from the time of placing in the cultures. Fresh weight and leaf length measurements and ascorbic acid estimations were made on each plant at the time of harvest.

(4) The Estimation of ascorbic acid.

The difficulties associated with the estimation of ascorbic acid in plant material have been discussed by several workers, Marston, 1942, Mapson, 1954, and Schaffert and Kingsley, 1955. Two methods were tried to assess their applicability to tomato plant tissue.

(a) Mapson's modification of the titration of ascorbic acid with the redox dye 2:6 dichlorphenol indophenol, as described by Lugg, 1942. This method is based on the fact that at pH 0.6 there is no appreciable condensation between ascorbic acid and formaldehyde; compounds containing sulphhydryl groups on the other hand which would normally interfere with the titration, condense rapidly with

formaldehyde at this pH and so their interference is eliminated. At pH 2.0 formaldehyde condenses relatively rapidly with ascorbic acid but more slowly with the other main group of interfering substances, the "Reductones". Use is made of the linear rate of formaldehyde-reductone condensation; aliquots of plant extract buffered at pH 2.0 are titrated with indophenol at ten minute intervals. A graph of indophenol titre against time is plotted and extrapolated to zero time giving a "reductone correction factor" which is applied to the titre obtained at pH 0.6.

Tomato shoot extracts, particularly those from ammonia-grown plants, were found to be coloured pink, i.e. a colour very similar to that attained with the end point of the indophenol titration. This colouration, probably due to the presence of anthocyanins or allied pigments, could be removed by shaking the extract with a small quantity of Norite carbon. This procedure however, results in a loss of ascorbic acid which may be as much as 15%, and moreover is not consistent.

(b) The second method of ascorbic acid estimation attempted was that of Schaffert and Kingsley,

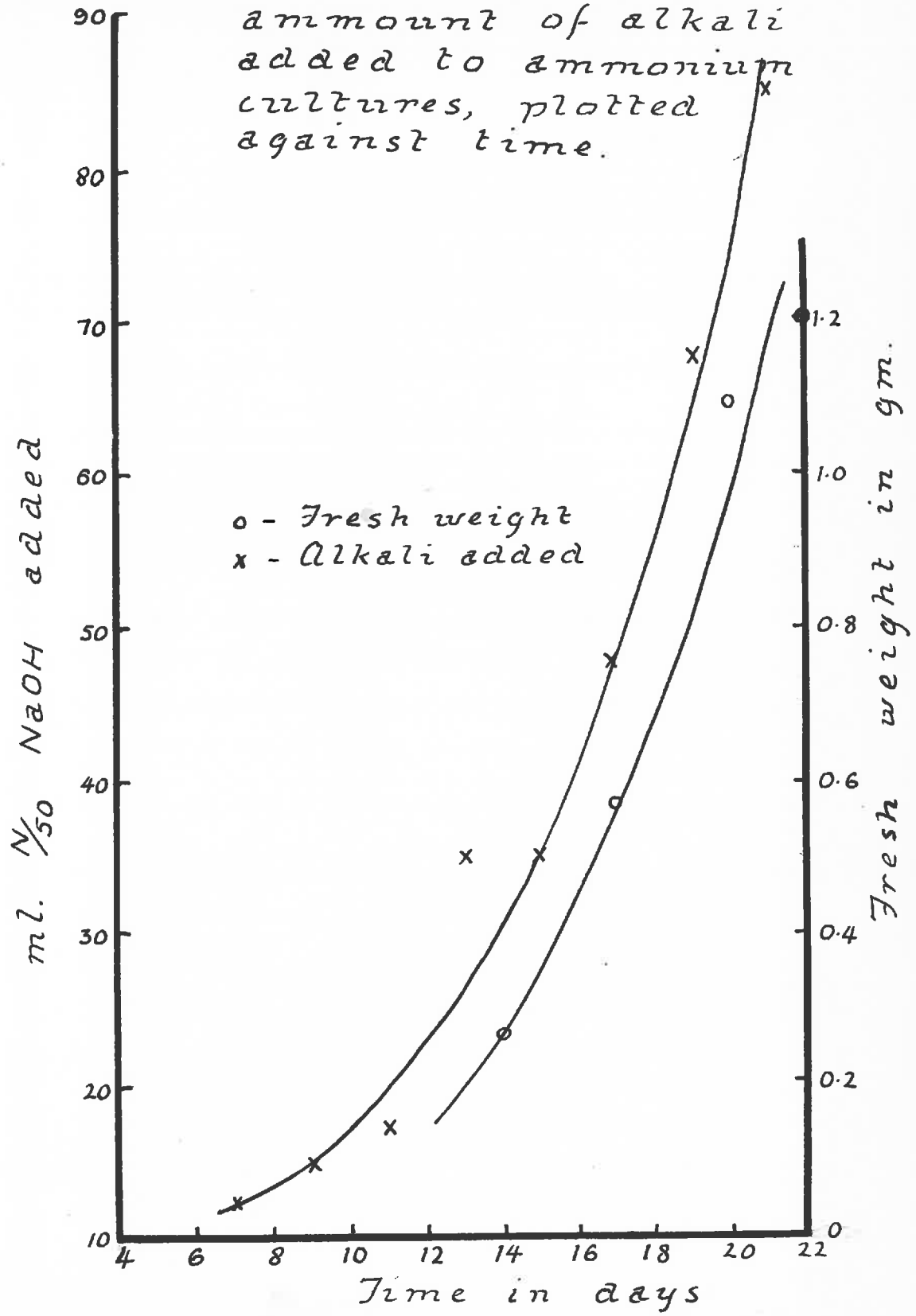
1955, based on the coupling of dehydroascorbic acid with 2:4 dinitrophenyl hydrazine to form a bis-2:4 dinitrophenylhydrazone; this hydrazone in the presence of concentrated sulphuric acid, undergoes a molecular rearrangement to form a red-coloured compound which can be estimated spectrophotometrically, having a maximum optical density at 540 m μ. Using this proceedure, known quantities of ascorbic acid added to tissue extracted in cold 5% metaphosphoric acid could be recovered to an accuracy of within 2%. This proceedure was therefore used to obtain the ascorbic acid values quoted in this experiment.

It must be noted at this point that in the experiments which follow that the ascorbic acid as estimated may reflect the proportion and form of the vitamin actually involved in metabolism at the time of analysis, but there is no evidence available to prove that this is so.

RESULTS:

In Figure 5 the fresh weight of plants and account of $\frac{N}{50}$ sodium hydroxide added to ammonium cultures are plotted against time on the same graph.

Figure 5 - Fresh weight of plants in ammonium culture and ammount of alkali added to ammonium cultures, plotted against time.



There is no significant difference in fresh weight between plants grown in complete, nitrate or ammonium culture solutions until after the twentieth day when the ammonia-grown plants begin to show a significantly smaller fresh weight. This result is not repeated in subsequent experiments when the divergence in size and fresh weight occurs at a much earlier stage. This may be related to the limiting light intensity at the time of the present experiment.

In Table VIII the ascorbic acid contents of tomato plants at different times of harvest are shown.

The only statistically significant point arising from the data in Table VIII is the lower absolute amount of ascorbic acid per plant in the ammonia cultures after 22 days, which reflects the difference in size of the plants, c.f. Table VII. The variability of the data prohibits any detailed inferences being made from these results. Of considerable interest in Table VIII is the fact that the ascorbic acid content per unit weight of tissue is not significantly different between nitrate and ammonia grown plants. This is contrary to the results of Mapson et al, 1949, using seedlings of Lepidium sativum, and of Sastry and Sarma, 1955, who provided evidence for the inhibition of ascorbic acid

TABLE VII. THE FRESH WEIGHTS IN GRAMS AT TIME OF HARVEST, OF PLANTS GROWN IN COMPLETE, NITRATE AND AMMONIUM CULTURES. EACH VALUE REPRESENTS THE MEAN WEIGHT OF SIX PLANTS WEIGHED.

Days to Harvest.	Mean Fresh Weight of Plant, in Grams. Complete Solution.	Nitrate Solution.	Ammonia Solution.
14	0.310	0.392	0.267
17	0.579	0.576	0.563
20	1.117	0.938	1.098
22	1.841	1.658	1.200

TABLE VIII. ASCORBIC ACID CONTENT OF PLANTS WHEN GROWN IN COMPLETE, NITRATE AND AMMONIA CULTURES. EACH VALUE REPRESENTS THE MEAN ASCORBIC ACID CONTENT OF SIX PLANTS ANALYSED SEPARATELY.

Days to Harvest.	Complete Solution.		Nitrate Solution.		Ammonia Solution.	
	Ascorbate per plant gms. 10^{-6}	Ascorbate per 100 gms. F.W. gms. 10^{-3}	Ascorbate per plant. gms. 10^{-6}	Ascorbate per 100 gms. F.W. gms. 10^{-3}	Ascorbate per plant gms. 10^{-6}	Ascorbate per 100 gms. F.W. gms. 10^{-3}
14	171.9	55.2	155.2	53.4	109.5	44.0
17	270.7	47.3	282.2	51.0	235.9	40.3
20	437.7	45.3	379.9	39.4	393.3	32.2
22	796.1	37.3	818.4	48.5	441.8	39.4

synthesis by ammonium ions; in both cases however, the experimentation is open to the criticism that single salt solutions were used, representing an "unphysiological" state of affairs and there is no mention of the pH's of the solutions used.

In Tables IX, X, XI and XII are recorded the leaf lengths of the plants at the time of harvest. Each value is the mean of six measurements.

The first true leaf, Table IX, continues to elongate at approximately the same rate in all three treatments up to the twentieth day, whereafter the first leaf of the ammonia grown plant ceases to grow, although there is a slower but continued elongation of the first true leaf of nitrate and complete culture grown plants. The younger third leaf, Table XI, begins to show a reduced elongation at an earlier stage in the ammonia cultures. Further, the fourth leaf, Table XII, is clearly visible after seventeen days in complete culture and nitrate culture grown plants but is absent from the ammonia grown plants, although at this time there is no difference in growth rates of leaves one and two between complete culture, nitrate and ammonia grown plants. If one takes leaf length as an index of growth, the growth of these first two true leaves could be largely at the expense of seed protein nitrogen (though this has not been established for the present conditions of experiment), and nitrate supplied in the seedling

TABLE IX. LENGTH OF FIRST TRUE LEAF, MEASURED IN CMS.

Time of Measurement. (Days)	Complete Solution.	Nitrate Solution.	Ammonia Solution.
14	4.01	4.27	3.2
17	5.2	4.8	4.2
20	6.7	6.0	6.2
22	7.1	6.7	6.2

TABLE X. LENGTH OF SECOND TRUE LEAF, MEASURED IN CMS.

Time of Measurement (days)	Complete Solution.	Nitrate Solution.	Ammonia Solution.
14	3.8	3.9	2.7
17	5.0	4.7	4.5
20	6.1	6.1	6.0
22	7.7	7.0	6.0

TABLE XI. LENGTH OF THIRD TRUE LEAF, MEASURED IN CMS.

Time of Measurement (days)	Complete Solution.	Nitrate Solution.	Ammonia Solution.
14	1.3	1.5	0.6
17	2.3	2.6	1.9
20	5.2	5.0	4.2
22	7.6	6.7	4.2

TABLE XII. LENGTH OF FOURTH TRUE LEAF, MEASURED IN CMS.

Time of Measurement (days)	Complete Solution.	Nitrate Solution.	Ammonia Solution.
14	-	-	-
17	0.7	0.6	-
20	2.8	2.6	1.9
22	5.2	4.2	2.3

dish; hence it might be supposed that if the reduced growth in ammonium cultures is associated with a diminishing supply of nitrate nitrogen, it might be expected to be in the younger, actively protein synthesising leaves that this reduced growth is first observed. Such an hypothesis, in terms of protein synthesis, must explain why, if enzyme protein is being continuously turned-over in all parts of the plant, should the nitrate become depleted and hence growth retarded, in the apical region first? Three distinct, though not mutually exclusive, suggestions might be made.

(i) In terms of adaptive enzyme formation: It might be supposed that nitrate, initially present in leaves one and two, though soon depleted was, while still present, responsible for the adaptive formation of enzyme proteins or their templates which persisted sufficiently to complete the development of these leaves, but could not be translocated to the new leaves;

(ii) In terms of translocation: It could be that ammonia or the organic compounds into which it is initially incorporated are unable to reach the growing regions at the same rate as nitrate and hence form a rate-limiting step for the formation of new leaves;

(iii) In terms of photosynthesis:- If as in case (i) seed reserve nitrogen and the seedling culture nitrate accounted for the similar growth of leaves one and two in all cultures but subsequent absence of nitrate in some way reduced the rate of photosynthesis, the consequence would be a small quantity of carbohydrate available for translocation which would restrict the growth of the younger leaves. The older leaves, one and two, of ammonia grown plants, producing a limited amount of carbohydrate which could be metabolised in situ, would not be affected so rapidly as the younger ones. These general hypotheses will be examined further, Chapter III, in relation to further experimental work.

EXPERIMENT IV. THE EFFECT OF MOLYBDENUM DEFICIENCY ON THE GROWTH AND ASCORBIC ACID CONTENT OF TOMATO PLANTS RECEIVING VARIOUS FORMS OF INORGANIC NITROGEN SUPPLY.

INTRODUCTION:

Under the conditions of Experiment III, the form of the inorganic nitrogen supply was without effect

on the ascorbic acid content of the plants when expressed in terms of a unit fresh weight of tissue. As previously discussed, molybdenum deficiency has been shown to result in a lowering of the ascorbic acid content of plants under certain conditions. It is the purpose of the following experiment to compare the effect of molybdenum deficiency on the growth and ascorbic acid content of plants growing in complete, all nitrate, and all ammonia culture solutions.

MATERIALS AND METHODS:

Plants.

Seedlings were raised in the manner described for Experiment III. Sommer, 1926, indicated the possibility that seeds of some plants might contain sufficient quantities of a particular microelement to carry them through their life cycle. This does not appear to be the case with molybdenum in the tomato, but there is a suggestion that the onset of deficiency symptoms will occur earlier in the life cycle if the molybdenum content of the seed is relatively low. For this reason tomato seed of the variety Pan American, supplied by Dr. Spencer, harvested from plants grown on soils low in molybdenum, were used.

Culture Solutions.

The culture solutions used were of the same composition as for Experiment III, the salts being puri-

fied to remove traces of molybdenum present as an impurity. Molar stock solutions of the macronutrients were purified free of molybdenum by recrystallisation, followed by co-precipitation with copper sulphide at pH 2 - 4, Stout and Meagher, 1948. Spectrographic analysis was used to determine the residual molybdenum in ferrous ammonium sulphate and sodium hydroxide. Steinberg, 1939, emphasised that, in the case of macronutrient salts, purity, as revealed by spectrographic analysis, might not be sufficiently sensitive to ensure that molybdenum deficiency conditions had been obtained. Spectrographic analysis of solid A.R. ferrous ammonium sulphate revealed no trace of molybdenum with detection guaranteed at 6.0 p.p.m., this would ensure less than 0.25×10^{-4} p.p.m. of molybdenum per culture from this source. On this evidence unpurified A.R. ferrous ammonium sulphate was used in the culture solutions as a source of iron. Hydrochloric acid for the adjustment of the pH of culture solutions was purified by redistillation before use. No method was readily available for the purification of sodium hydroxide free of molybdenum; however, a quantity of A.R. sodium hydroxide was neutralised with distilled hydrochloric acid and the sodium chloride so formed examined spectrographically for the presence of molybdenum. The analysis revealed no molybdenum with a detection limit of 4.0 p.p.m.; assuming an addition of 100 ml.

$\frac{N}{50}$ sodium hydroxide per culture this would represent an addition of not more than 1.4×10^{-4} p.p.m. of molybdenum per culture from this source. No purification of micronutrients was necessary because of the small quantities used in a culture solution.

Ascorbic acid estimation.

Ascorbic acid was estimated by the method of Schaffert and Kingsley as in Experiment III.

Experimental design and harvesting.

Six cultures (24 plants) of each treatment, i.e., complete culture solution, complete culture solution minus molybdenum, all ammonia culture solution, and all ammonia culture solution minus molybdenum, were grown, and one plant removed from each culture vessel at four intervals, 14, 17, 20 and 22 days from the time of placing in the cultures. Fresh weight and ascorbic acid estimations were made on each plant at the time of harvest.

RESULTS:

The first signs of molybdenum deficiency appeared at twelve days from the time of placing the plants in the culture solutions. The affected plants developed a marked chlorosis of the older leaves with a normal green colour persisting along the veins. Young leaves were not visibly affected by the deficiency. In a period of bright sunshine on the thirteenth day, the symptoms of molybdenum

deficiency became rapidly more severe; previously slightly chlorotic regions showed a "clearing" and subsequent collapse of the tissue within a single afternoon. This is an extreme case of the more general observation by several workers, that under ordinary greenhouse conditions it is easier to obtain symptoms of molybdenum deficiency in summer than in winter. Whether this effect is a consequence of temperature or light intensity or both and whether the effect is primarily upon the rate of re-distribution of molybdenum within the tissues or upon other metabolic reactions, is not known. An inspection of the root systems after twelve days revealed a difference between the roots of plants receiving molybdenum and plants not receiving molybdenum, when grown on otherwise complete culture solution; these may be summarised as on page 83 of this thesis.

At no time were symptoms of molybdenum deficiency obtained in ammonia grown plants. The most likely reason for this is the presence of molybdenum in the caustic soda used for maintaining the pH of the ammonia cultures. A further possibility is that the molybdenum requirement is lower in the presence of ammonia than of nitrate and that this lower level has not been achieved in the purification of solutions, c.f. Mulder, 1948. Again it may be that under conditions of lower growth rate of the ammonia grown plant a redistribution of molybdenum

	Complete Culture.	Complete Culture minus Molybdenum.
1.	Mean length of root system 20 cms.	Mean length of root system 13 cms.
2.	Root bearing moderate number of laterals.	Root bearing large number of laterals.
3.	Laterals hanging limp on removal of root from culture solution.	Laterals "spikey", i.e. standing out rigidly when root was removed from solution.
4.	Overall colour of root system white.	Overall colour of root system pale brown.
5.	Root apices greyish white.	Root apices yellowish brown.

is possible which cannot be maintained in the more rapidly growing plant receiving nitrate nitrogen. A perhaps less likely reason for the failure to obtain molybdenum deficiency in ammonia grown plants would be the existence of the situation alleged to exist in Chlorella, Arnon et al, 1955, and Ichioka et al, 1955, where the molybdenum requirement was entirely replaceable by ammonia nitrogen, this explanation is rendered the more unlikely by the at least initial presence of ammonia in the complete culture solution.

Tables XIII and XIV show the ascorbic acid content of plants receiving the different treatments.

At 17, 20 and 22 days the amount of ascorbic acid present in plants growing in complete culture minus molybdenum is, when expressed in this way, significantly lower than in similar plants receiving molybdenum or in ammonia-grown plants, whether receiving molybdenum or not. There are no further significant differences between treatments.

After 17, 20 and 22 days the amount of ascorbic acid present per plant is significantly lower in plants grown in complete cultures minus molybdenum than in similar plants receiving molybdenum or in ammonia-grown plants whether receiving molybdenum or not. Between the twentieth and twenty-second days there is a marked decline in the growth rate and ascorbic acid content of

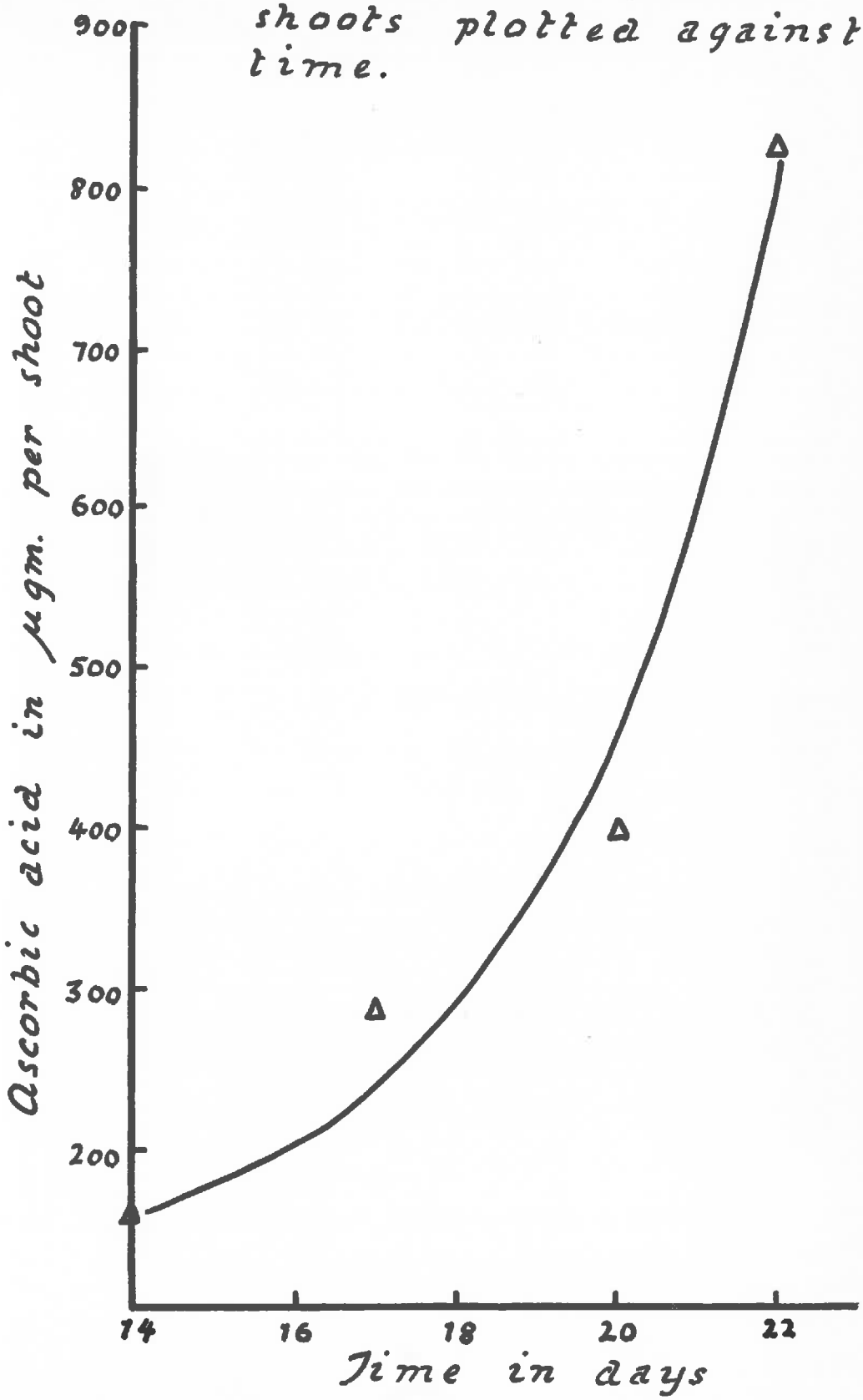
TABLE XIII. THE ASCORBIC ACID CONTENT OF WHOLE TOMATO SEEDLINGS RECEIVING AMMONIA OR BOTH AMMONIA AND NITRATE NITROGEN. DATA EXPRESSED AS MGMS. ASCORBIC ACID PER 100 GMS. FRESH WEIGHT. EACH VALUE IS THE MEAN OF SIX PLANTS ANALYSED.

Time in Days.	Complete Culture.	Complete Culture. minus Molybdenum.	Ammonium Culture.	Ammonium Culture minus Molybdenum.
14	56.2	49.5	42.0	48.4
17	45.1	34.7	43.0	40.5
20	43.1	28.0	35.3	37.3
22	36.0	27.8	35.5	34.1

TABLE XIV. THE ASCORBIC ACID CONTENT OF WHOLE TOMATO SEEDLINGS RECEIVING AMMONIA OR BOTH AMMONIA AND NITRATE NITROGEN. DATA EXPRESSED AS MICROGRAMS PER PLANT. EACH VALUE IS THE MEAN OF SIX PLANTS ANALYSED.

Time in Days.	Complete Culture.	Complete Culture. minus molybdenum.	Ammonium Culture.	Ammonium culture minus molybdenum.
14	160.0	125.5	117.3	120.1
17	288.7	196.6	285.3	265.3
20	400.2	159.0	388.4	381.6
22	820.5	217.7	501.0	478.2

Figure 6 — Ascorbic acid content of shoots plotted against time.



ammonia grown plants, the increasing difficulty of controlling the pH of the ammonium cultures at this stage however detracts from any significance which might be attached to this result.

The graph of ascorbic acid content of plants plotted against time, Figure 6, does not run parallel to the growth (i.e. fresh weight - time) curve, this is possibly due to the fact that the ascorbic acid content of different parts of the plant varies greatly (Table XV) and the ratio of the weights of these different parts, e.g. root : stem : leaves, changes rapidly in the young plant.

EXPERIMENT V. The effect of AMMONIUM IONS ON THE GROWTH OF TOMATO PLANTS DEFICIENT IN MOLYBDENUM.

INTRODUCTION:

Experiment IV failed in attempting to determine the effect of molybdenum deficiency on the ascorbic acid content of ammonia-grown plants, probably owing to molybdenum contamination in the sodium hydroxide used for adjusting the pH of the cultures. There appears to be no satisfactory method available for purifying sodium hydroxide free of traces of molybdenum. In an attempt to obviate this difficulty plants were grown on nitrate culture deficient in molybdenum and then ammonium chloride added to the culture in order to find whether the ammonium

TABLE XV. THE ASCORBIC ACID CONTENT OF DIFFERENT PARTS OF THE TOMATO PLANT AT 24 DAYS. EACH VALUE IS THE MEAN OF THREE PLANTS.

Tissue analysed.	Fresh weight. gms.	Absolute amount of Ascorbic acid present, micrograms.	Ascorbic acid Mgms./100 gms. fresh weight.
Cotyledons plus true leaves, 1, 2, 3.	1.870	387.4	21.97
True leaves, 4, 5.	1.837	978.4	54.22
Apex and developing leaves.	0.585	442.5	85.24
Stem.	2.961	281.9	9.38
Root.	0.495	36.3	7.34

ion could stimulate formation of ascorbic acid in molybdenum deficient tissue, in the time interval before the pH became low enough to have a deleterious effect on growth.

It might be argued that the results of this experiment could be infered without the experiment ever being done since it has been shown that molybdenum deficiency has similar effects on fresh weight and ascorbic acid content of tomato plants grown on both all-nitrate and in complete culture solutions. If therefore, ammonia permitted a by-passing of the molybdenum deficiency - induced nitrate reductase block, and hence more normal pathways of metabolism in which ascorbic acid could be synthesised, as is suggested by the work of Virtanen, and Seubert-von Hausen, referred to earlier, then one would have expected molybdenum deficient plants growing in a complete culture solution which includes nitrate and ammonium nitrogen to have yielded a greater fresh weight and higher ascorbic acid content, than plants growing in an all-nitrate culture solution. However, at about this time checks were made on the ammonium status of culture solutions at different time intervals in the course of an experiment, and it was found that the ammonia loss from the culture solution could not be completely accounted for by the nitrogen taken up by the plants. This observation led to an investigation of the ammonia loss from an aerated culture solution on standing, Chapter II,

Experiment VI; frequent renewal of the ammonia was found to be necessary to compensate for the loss of gaseous ammonia to the atmosphere.

MATERIALS AND METHODS:

Plants.

Seeds were germinated and the young plants grown as described for the previous experiment.

In the absence of further supplies of tomato seed from plants grown on soils low in molybdenum, recourse was made to commercial seed of variety Bonny Best. A strong suggestion of the effect of residual molybdenum in the parent seed comes from the observation that using the same batch of purified salts as for the previous experiment, the onset of molybdenum deficiency was slower (14 - 18 days), and more erratic using commercial seed than the sample provided by Dr. Spencer.

Culture Solutions.

The purification of salts and composition of culture solutions was as described for Experiment IV.

Analyses.

Ascorbic acid was estimated by the method of Schaffert and Kingsley, previously described.

Free ammonia in the tissues was estimated by a modification of the method of Kobel, Mayer and Gottfried, 1944. Each plant was ground in a pestle and mortar with

a 15% W/V solution of NaCl, and the extract made up to 100 ml. The extract was then centrifuged at 400×g. for ten minutes; 40 mls. of the supernatant were placed in a tube in an aeration train and 5.0 mls. of a solution of 5% sodium borate in 0.5N. NaOH added. Two drops of octyl alcohol were then added to prevent frothing. The reaction tube was connected to an aeration train with an acid trap for atmospheric ammonia in front and a collecting tube containing 1.5 ml. 2% boric acid to collect the ammonia released. The ammonia liberated was then determined by back-titration with N/50 hydrochloric acid.

Experimental design and Harvesting.

Twenty-four cultures, each containing four plants were divided equally between three treatments and placed in random sequence in the greenhouse. After fourteen days deficiency ∧ an interveinal chlorosis characteristic of molybdenum was developed but the symptoms were not equally severe from one culture to the next, probably due to residual molybdenum in some seeds. On the fourteenth day one plant was taken from each culture and the fresh weights and ascorbic acid contents determined. Similar harvests were taken at 15, 18 and 21 days. At the final harvest, 21 days, only four plants from each treatment were analysed for ascorbic acid; the remaining four being used for free ammonia estimations. The three treatments used were:-

1. Eight nitrate cultures with molybdenum present.
2. Eight nitrate cultures with molybdenum absent.

3. Eight nitrate cultures deficient in molybdenum with 0.442 gms. ammonium chloride (0.0036N) added to each culture at the time of the first harvest on the fourteenth day.

RESULTS:

The change in fresh weight of plants in the course of the experiment is recorded in Table XVI.

Plants deficient in molybdenum are of significantly lower fresh weight than plants receiving molybdenum, after eighteen days. Addition of ammonium nitrogen to cultures of nitrate-grown plants deficient in molybdenum resulted in no significant increase in fresh weight.

The uneven intensity of molybdenum deficiency symptoms introduced a high variability into the data. The ascorbic acid content of molybdenum deficient plants was significantly (5% level) less than that of normal plants after fifteen days. Addition of ammonium nitrogen resulted in no statistically significant increase in the ascorbic acid content of the plants. See also Table XVIII.

After two days, plants which were fed ammonium nitrogen showed a blueing and curling of the leaves. By the final harvest, seven days after the time of feeding ammonia, these symptoms had become more acute along with an increasing severity of the interveinal chlorosis, this

TABLE XVI. FRESH WEIGHT IN GRAMS OF PLANTS AT EACH HARVEST. EACH VALUE IS THE MEAN WEIGHT OF EIGHT PLANTS.

Time in Days.	Fresh weight in gms. of all nitrate grown plants.	Fresh weight in gms. of all nitrate plants deficient in molybdenum.	Fresh weight in gms. of molybdenum deficient plants fed NH_4 at 14 days.
14	0.804	0.750	0.732
15	0.982	0.847	0.851
18	2.363	1.212	1.300
21	3.979	1.987	2.066

TABLE XVII. THE CHANGE IN ASCORBIC ACID CONTENT OF PLANTS, EXPRESSED AS MGMS. ASCORBIC ACID PER 100 GMS. FRESH WEIGHT. EACH VALUE REPRESENTS THE MEAN ASCORBIC ACID CONTENT OF EIGHT PLANTS ANALYSED.

Time in Days.	Ascorbic acid content of all-nitrate grown plants.	Ascorbic acid content of all nitrate grown plants deficient in molybdenum.	Ascorbic acid content of molybdenum deficient plants fed NH_4 after 14 days.
14	60.5	49.1	52.1
15	55.3	39.1	43.8
18	44.6	30.3	31.6
21	37.3	28.1	31.5

TABLE XVIII. THE CHANGE IN ASCORBIC ACID CONTENT OF PLANTS, EXPRESSED AS MICROGRAMS ASCORBIC ACID PER PLANT. EACH VALUE REPRESENTS THE MEAN ASCORBIC ACID CONTENT OF EIGHT PLANTS ANALYSED.

Time in Days.	Ascorbic acid content of all-nitrate grown plants.	Ascorbic acid content of all nitrate grown plants deficient in molybdenum.	Ascorbic acid content of molybdenum deficient plants fed NH_4 after 14 days.
14	486.4	368.3	381.4
15	545.1	331.2	392.7
18	1053.9	367.2	410.8
21	1484.2	558.3	650.8

was shown to be accompanied by high levels of free ammonia within the tissues, Table XIX.

The level of free ammonia in the molybdenum deficient plants grown on nitrate is significantly lower than in plants from the other two treatments, suggesting that the ammonia present in the plants receiving molybdenum might be formed in metabolism. There is however, no good evidence for this, the ammonia could be formed during the extraction process, by either reduction or lysis of labile intermediates. The level of ammonia in plants fed ammonium chloride at 14 days probably represents a toxic concentration; this might be a consequence of a lowering of the pH of the culture solution resulting in a damaging of the roots so that the restraint on the entry of ammonium ions was reduced. Alternatively, it is possible that a lowered concentration of an appropriate carbohydrate skeleton for the incorporation of ammonia might account for its accumulating in the free state; there is not however, satisfactory data available to show that carbohydrates are in fact depleted in molybdenum deficient tissues. Again it is possible that molybdenum is essential for the incorporation of ammonia into organic combination. Four main pathways are so far known, mainly from in vitro studies, through which ammonia may enter into organic combination in the plant; they are mediated by the enzymes glutamic dehydrogenase, carbamyl phosphate synthetase; glutamine synthetase and those converting ornithine to arginine. None

TABLE XIX. THE FREE AMMONIA CONTENT OF PLANTS AFTER 21 DAYS, EACH VALUE IS THE MEAN OF FOUR ANALYSES.

Treatment.	Fresh weight of plant in gms.	Free ammonia in mgms. NH_3 per 10 gms. fresh weight?
All-nitrate grown plants.	3.905	0.208
All nitrate minus Molybdenum.	1.917	0.082
All nitrate minus Molybdenum plus NH_3 at 14 days.	1.983	2.679

of these enzymes are known to require molybdenum for their functioning.

Whether these possible explanations of the failure of ammonia to replace molybdenum are correct or not, the general conclusion from this experiment, that added ammonia cannot bye-pass the molybdenum requiring step in nitrate reduction and hence mediate the biosynthesis of ascorbic acid, is probably a valid one.

EXPERIMENT VI. THE EFFECT OF ASCORBIC ACID ON REDUCTION OF NITRATE BY CUT TOMATO SHOOTS.

INTRODUCTION:

At the beginning of Chapter I reference was made to the work of Virtanen and Saubert von Hausen, 1939; it may be that in these experiments molybdenum was deficient, as no reference to the element is made. If this were so, and if molybdenum is essential for some step in the biosynthesis of ascorbic acid, then the effect of ascorbic acid in promoting the growth of plants receiving all their nitrogen in the form of nitrate might be due to the vitamin alleviating the molybdenum deficiency. Such an explanation is however, rendered less likely since in the same series of experiments, ammonia did promote good growth of cotyledless seedlings in the absence of added ascorbic acid. To this point, however, it may be argued that there was enough molybdenum present to support metabolism from the stage of ammonia formation but insufficient for nitrate reduction. Against this on the other hand it is perhaps unlikely that the different

molybdenum-requiring processes should be mutually exclusive at low levels of molybdenum, resulting in almost no growth at all on nitrate without added ascorbic acid, as was Virtanen and von Hausen's observation.

If one therefore rules out the possibility of molybdenum deficiency being the cause of Virtanen and von Hausen's results one must look for other ways in which ascorbic acid may influence the utilisation of nitrate; of particular interest would be to know whether the vitamin is concerned in the process of reduction from nitrate to ammonia. As a preliminary to such a study, an attempt was made to widen the scope of Virtanen and von Hausen's experiments by enhancing the ascorbic acid content of tomato shoots, and looking for an effect on the amount of nitrate in the leaves.

Attempts were made to feed ascorbic acid through intact tomato leaves by direct immersion of the leaf in solutions of sodium ascorbate. It was found, however, that sodium ascorbate even at relatively high concentrations would not pass through the intact leaf surface. Hewitt, 1954, employed a method of ascorbic infiltration through a cut surface of the leaf. It was found however, that this method does not give an even distribution of the ascorbic acid throughout the shoot. Sodium ascorbate is readily taken up by cut shoots of tomato when the cut stem is placed in a solution of the vitamin, and this procedure for introducing the vitamin was adopted.

MATERIALS AND METHODS:

Plants.

Tomato plants var. Bonny Best were grown in complete culture solutions by the method previously described.

Treatments.

At 9.30 a.m., 21 days from the time of placing the seedlings in the culture solutions, the shoots of 24 plants were cut at the base under water. Twelve of the cut shoots were rapidly transferred to seperate beakers each containing 100 mls. of dilute culture solution, whilst the remaining twelve were placed each in a beaker containing 100 mls. M/50 sodium ascorbate at pH 5.6. The cut shoots were placed in diffuse daylight on the laboratory bench and at intervals between 0 - 9 hours, four plants from each treatment were harvested.

Analyses.

The leaf laminae from two of the harvested plants from each treatment were analysed for ascorbic acid content by the method of Schaffert and Kingsley, previously described, and the leaf laminae of the remaining two plants from each treatment were analysed for nitrate content by the method of Jones and Underdown, 1954. This method of nitrate estimation yielded consistently approximately 90% of nitrate added to tomato leaf homogenates.

RESULTS:

That ascorbic acid is readily taken up into the leaves through cut stems is seen from the data in Table XX, and Fig. 7.

The level of ascorbic acid in the leaves of plants placed in dilute culture solution remained almost constant throughout the nine hours experimental period. The level of ascorbic acid in the leaves of plants placed in sodium ascorbate solution increased in a manner approximately linear with time, being slightly more rapid in the earlier part of the experiment. Analyses of residual ascorbic acid in the beakers were not made, so that it is not known whether these analyses reflect the amount of ascorbic acid taken up by the shoots or contain a constant or fluctuating ascorbic acid catabolism component.

Changes in nitrate content of the leaves proved variable and were of the same order of magnitude as the experimental error, Table XXI and Fig. 8.

This experiment is preliminary, and poses many questions which would have to be answered before it could be satisfactorily repeated. Does proteolysis occur in cut shoots of tomato plants over the time period used? Although ascorbic acid is taken up into the leaves of cut shoots is there any guarantee that it penetrates to the site(s) in the cell where it is utilised? Is nitrate reduction limited by other factors such as the level of nitrate available at sites in the cell where it is reduced

TABLE XX. ASCORBIC ACID CONTENT IN MGMS. PER 100 GMS. FRESH WEIGHT, OF LEAVES FROM CUT SHOOTS OF 21 DAY OLD PLANTS PLACED IN DILUTE CULTURE SOLUTION AND IN N/50 SODIUM ASCORBATE SOLUTIONS. EACH VALUE IS THE MEAN OF TWO SAMPLES ANALYSED.

Time in Hours.	Shoots in dilute culture solution.	Shoots in N/50 sodium ascorbate solution.
0	38.1	27.0
1	39.6	68.4
3	37.8	114.9
5	40.3	156.5
7	41.0	186.4
9	40.2	217.3

Figure 7 — Uptake of ascorbic acid by cut shoots of tomato.

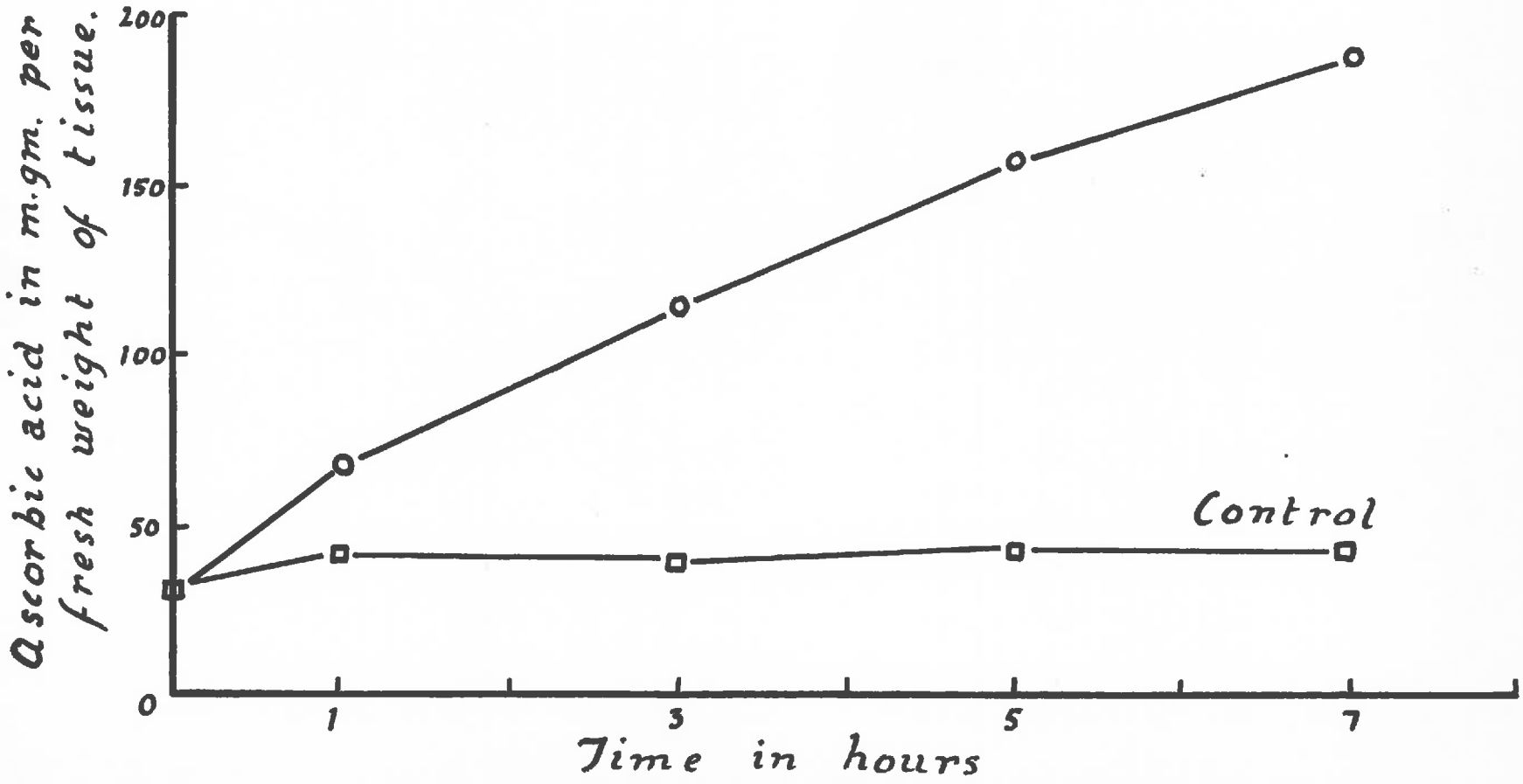
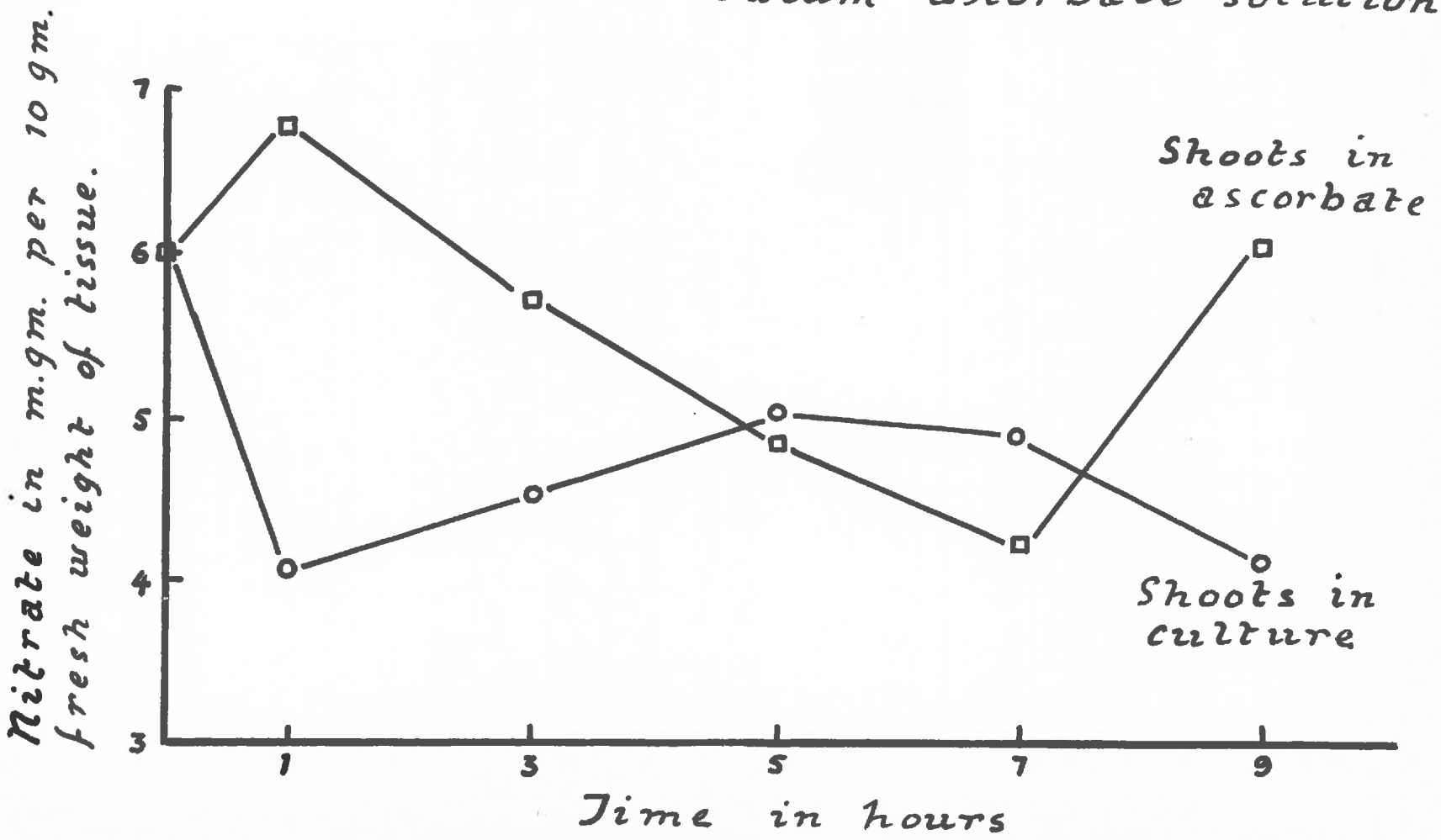


TABLE XXI. NITRATE CONTENT IN MGMS. PER 10 GMS. FRESH WEIGHT, OF LEAVES FROM CUT SHOOTS OF 21 DAY OLD PLANTS PLACED IN DISTILLED WATER AND IN N/50 SODIUM ASCORBATE SOLUTIONS. EACH VALUE IS THE MEAN OF TWO SAMPLES ANALYSED.

Time in Hours.	Shoots in distilled water.	Shoots in N/50 Sodium ascorbate solution.
0	6.21	5.70
1	4.03	6.83
3	-	5.74
5	5.01	4.83
7	4.88	4.22
9	4.11	6.80

Figure 8 — Nitrate contents of cut shoots placed in dilute culture solution and in sodium ascorbate solution.



and the amount of reduction which has occured in the period immediately prior to the cutting of the shoot. Does the excision of the root remove an essential source of co-factors to nitrate reduction or sink for products of reduction, either of which might be essential to the maintenance of reducing conditions in the leaves? Does the light regime prior to or during the ascorbic acid feeding influence the rate of nitrate reduction? From Experiments I - IV however, there arises a further problem perhaps more fundamental in the problem of higher plant nitrogen metabolism. From the data of Virtanen and Seubert-von Hausen, 1949, growth (as measured by dry weight and shoot length) of wheat seedlings from which the endosperm had been removed after one hour of soaking, when fed with nitrate and ascorbic acid, was as great as that of seedlings receiving ammonium nitrogen and no ascorbic acid. In the experiments with tomato seedlings, except at low light intensities, growth in ammonium cultures is never as great as when nitrate forms a whole or part of the inorganic nitrogen source. Chapters II and III are concerned with a description of some of the differences between nitrate and ammonia grown plants and an investigation of certain of the metabolic differences underlying them.

C H A P T E R I I

THE PROBLEM OF AMMONIUM CULTURES AND THE DIFFERENCES IN GROWTH AND STRUCTURE OF TOMATO PLANTS GROWN IN NITRATE AND AMMONIUM CULTURES.

In the course of preliminary experiments to decide the composition of ammonium cultures most favourable for growth of young tomato plants, there was found to be a marked discrepancy between the sum of the nitrogen content of the plants plus the residual nitrogen content of the culture solution, and the amount of nitrogen initially present in the culture solution. Some nitrogen may have been lost by incorporation into microorganisms in the culture solution and subsequent sedimentation. There was little evidence of sediment formation in the culture vessels. A further possibility is the loss of gaseous ammonia from the culture solutions; this was checked experimentally.

EXPERIMENT VII. THE LOSS OF GASEOUS AMMONIA FROM SOLUTIONS OF AMMONIUM SULPHATE ON STANDING AND FROM AERATED CULTURE SOLUTIONS UNDER GREENHOUSE CONDITIONS.

MATERIALS AND METHODS:

Fifty ml. samples of freshly made up solutions of ammonium sulphate were placed in 50 ml. beakers 20 cms. apart in a constant temperature room at 25° C. The area

of free water surface in each beaker was approximately 16.0 square centimetres. A trace of sulphanilamide was added to each beaker to prevent bacterial growth; a control test revealed that the sulphanilamide did not decompose to make any contribution to the ammonia estimations. Plating tests, made after 24 and 48 hours revealed little bacterial contamination in the beakers. Two concentrations of ammonium sulphate, 0.002M and 0.0004M, were prepared. For each concentration sets of four beakers were brought to initial pH's of approximately 5.0, 7.0 and 8.0 by addition of appropriate amounts of N/10 sodium hydroxide. At eight hourly intervals the beakers were weighed, and appropriate quantities of ammonia-free distilled water added to maintain the total volume of each beaker at 50.0 mls. At intervals of 0, 24, 48, and 72 hours, the pH of each solution was measured at a glass electrode; small samples were removed from each beaker, and the ammonia content determined by the micro-diffusion method of Conway.

RESULTS AND DISCUSSION:

The change in pH of the two solutions are shown in Figures 9a and 10a. It is seen that with both solutions there is a relatively rapid fall in pH over the first 24 hours, in fact of course the fall may have been more rapid since there was no measurement before the twenty-four hours. Figures 9 and 10 clearly show that for both solutions the loss of nitrogen from the solution follows the shift in pH.

Figure 9— Percentage loss of ammonia from solutions of ammonium sulphate on standing.

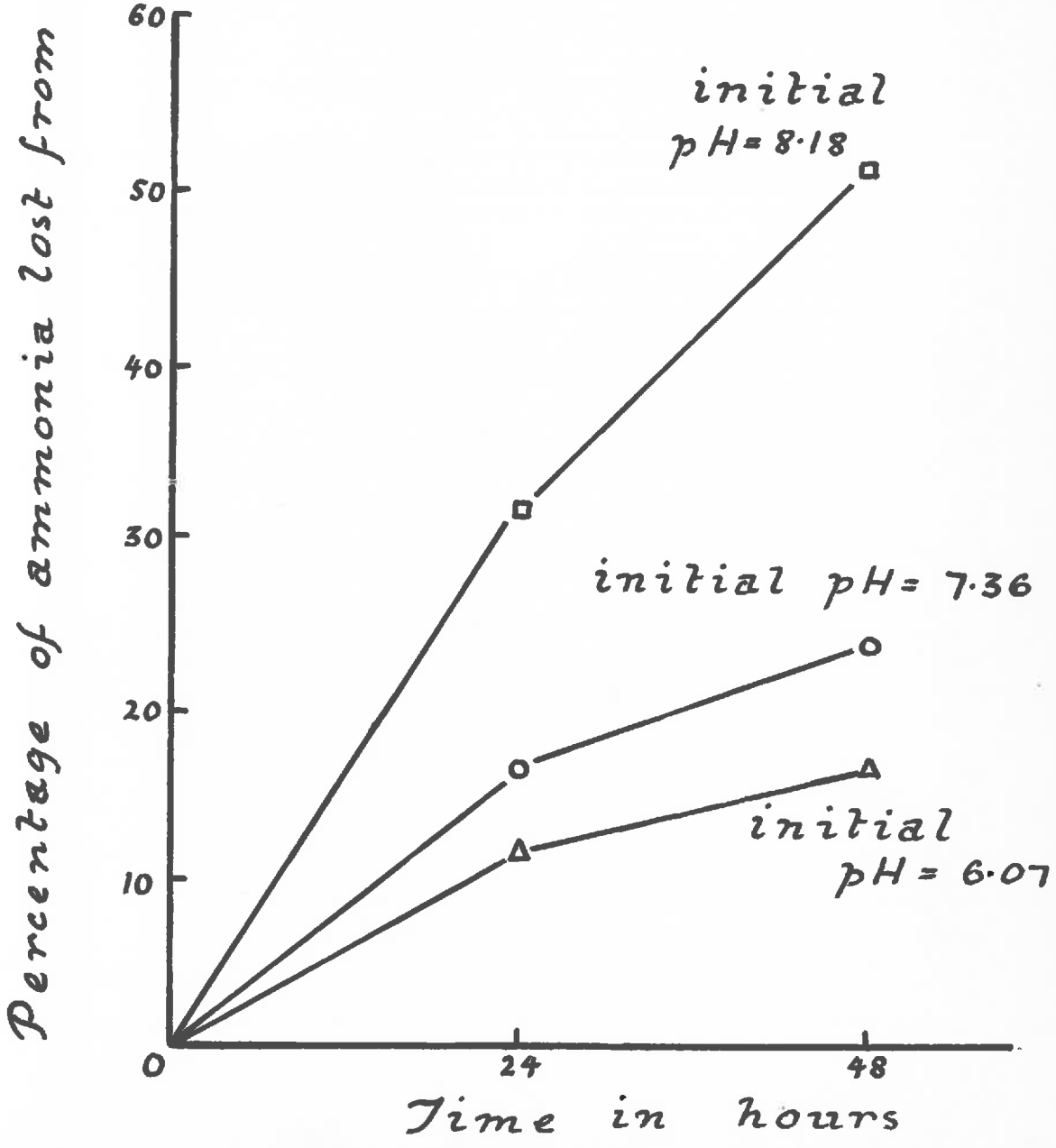


Figure 9a — Changes in pH of solutions of ammonium sulphate on standing.

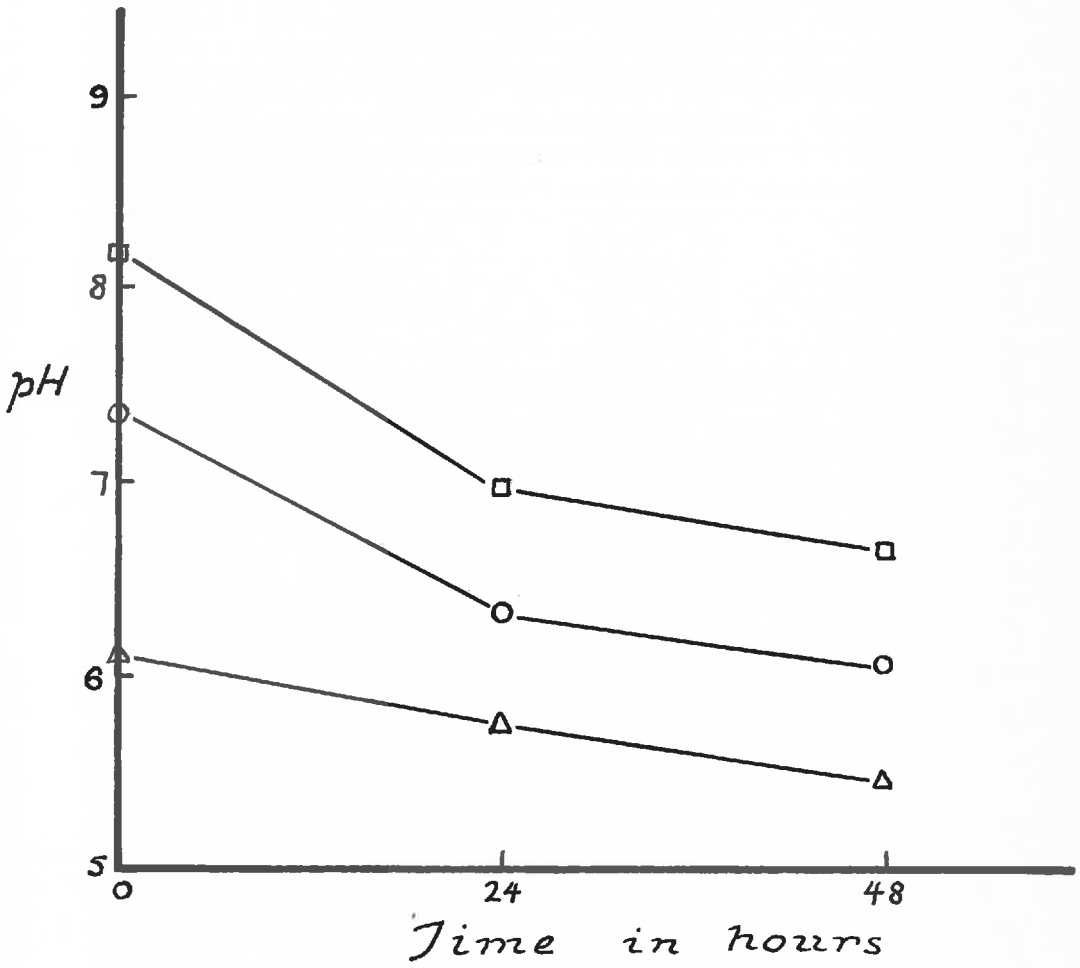


Figure 10 – Percentage loss of ammonia from solutions of ammonium sulphate on standing.

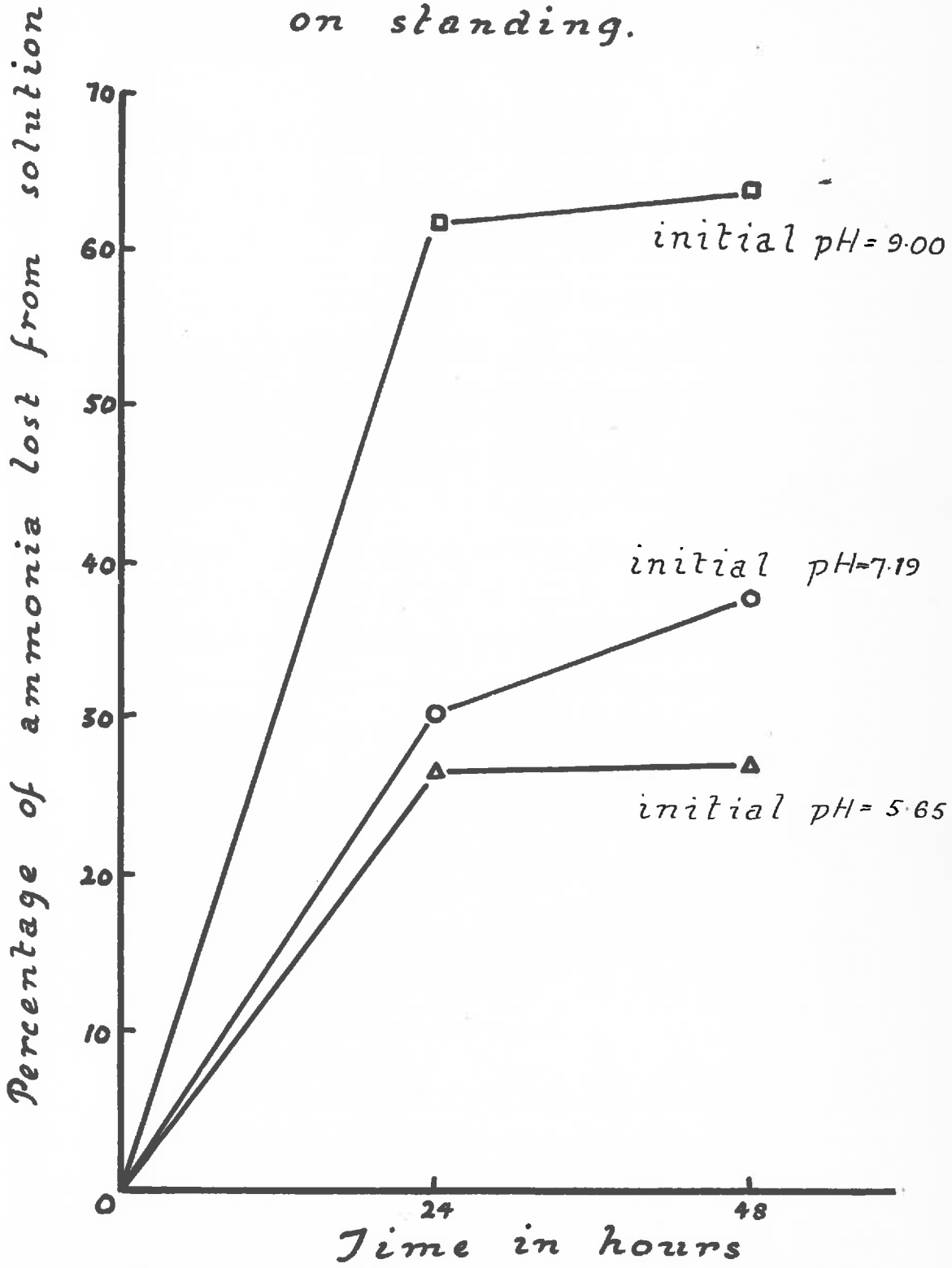
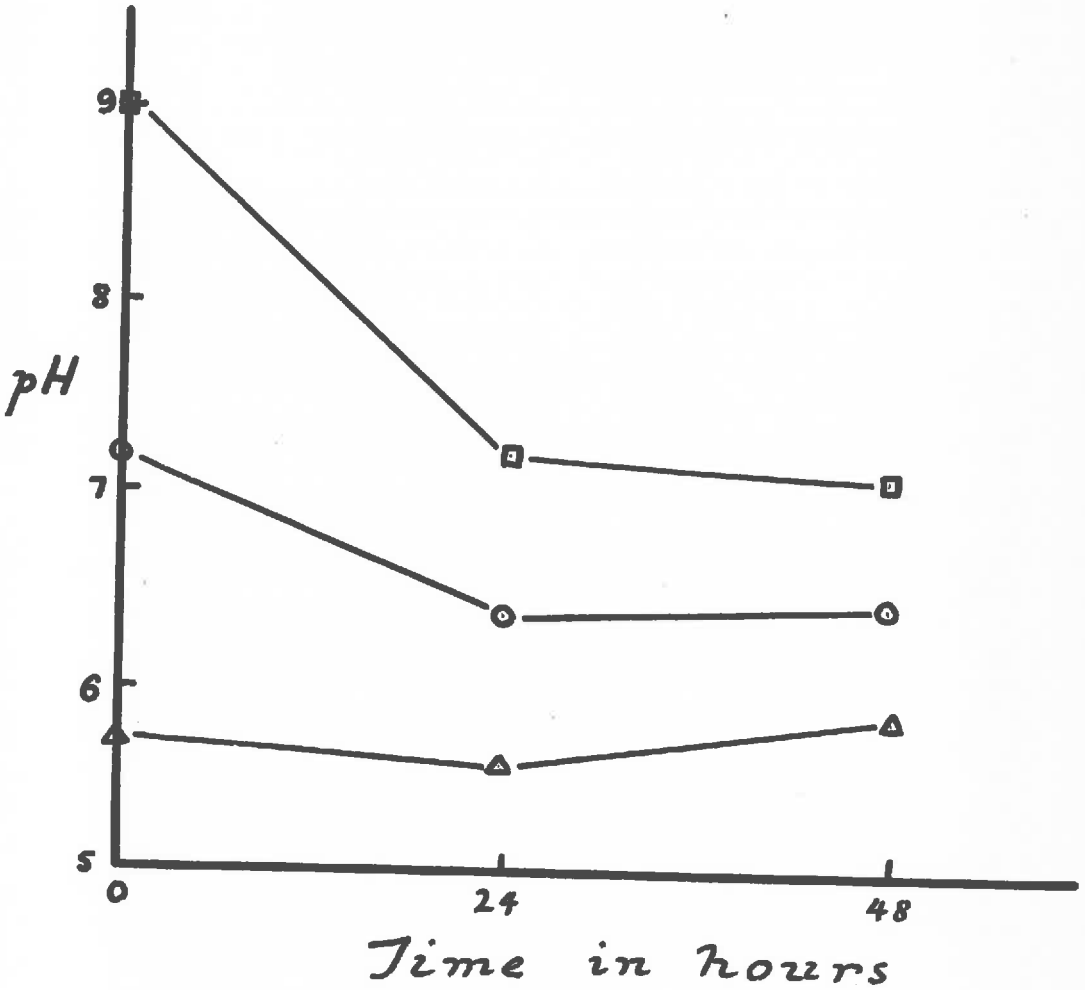


Figure 10a – Changes in pH of solutions of ammonium sulphate on standing.



It must be noted however, that the amount of dissolved carbon dioxide will also affect the pH, so that the change in pH will not necessarily be proportioned to the amount of ammonia given off.

It is seen that at pH 7.0 from 30 - 40% of the dissolved ammonia may be lost to the atmosphere within three days under these conditions. From the chemical standpoint this experiment is but approximate and clearly open to criticism, it serves however, to indicate that ammonia may be readily lost from free solutions and suggests that in a great many water culture experiments which contained initially both nitrate and ammonium nitrogen and were not frequently renewed, what is being studied after a relatively short time is growth on nitrate and not on both nitrate and ammonium nitrogen. It would seem that only a general suggestion as to why this ammonia loss should occur, can be given.

Henry's Law states that the mass of a gas dissolved by a given volume of a liquid is proportional to the pressure of the gas, at constant temperature.

i.e. $m = K.p$

where m = mass of gas dissolved.

p = pressure of the gas.

Let Cg = the gas concentration in the gaseous phase,

and Cl = the gas concentration in the liquid phase.

whence $K^1 = \frac{Cg}{Cl}$ (1)

For a solution such as ammonia in water, the law appeared not to hold, until Nernst, 1891, pointed out that a substance could not be regarded as distributed between two phases if its molecular condition in the two phases is different. However, if the molecules are associated in one phase but there exists an equilibrium between the associated and the unassociated molecules in that phase, then equilibrium will exist between the unassociated molecules in the two phases.

Thus in the case of ammonia and water we may write:

$$NH_3 \rightleftharpoons NH_3 + H_2O \rightleftharpoons NH_4OH$$

gaseous phase. liquid phase.

and if dissociation occurs there will be the further equilibrium

$$NH_4OH \rightleftharpoons NH_4^+ + OH^-$$

liquid phase.

In order to apply Henry's Law therefore, the constants for these equilibria must be known, so that the concentration of NH_3 in each phase may be calculated from the total concentration present.

If C_B = concentration of solvent molecules (water)

and C_A = concentration of ammonium hydroxide molecules

From the Law of mass action $\frac{C_L \times (C_B)^2}{C_A} = K1$ (11)

C_B is large and does not vary greatly in dilute solutions whence $(C_B)^2$ is constant so that the law of mass action becomes

$$\frac{C_L}{C_A} = K_2 \quad \text{(III)}$$

$$\frac{C_A + C_L}{C_L} = \frac{1 + K_2}{K_2} \qquad (iv)$$

Dividing equation (iv) into equation (ii)

$$\frac{C_g}{C_L + C_A} = \frac{K_1 K_2}{1 + K_2} = K_3 \qquad (v)$$

If C = total ammonia concentration in the liquid phase and α the degree of dissociation.

the law of mass action becomes $\frac{(C\alpha)^2}{C(1-\alpha)} = \frac{C\alpha^2}{1-\alpha} = K_4$

$$\therefore \alpha = \frac{-K_4 + \sqrt{K_4^2 + 4K_4C}}{2C}$$

Now $C_A = 1-\alpha$ of the total concentration C

Thus $K = \frac{C_g}{C_L} = \frac{C_g}{C(1-\alpha)} = \frac{2C_g}{2C + K_4 - \sqrt{K_4^2 + 4K_4C}}$

Calingaert and Huggins, 1923, applied this analysis to solutions of ammonia over a concentration range of 0.004 to 1.25 gms. per litre at 100° C. and found that when the correction for dissociation is made the agreement with Henry's Law is good.

If one now considers an aqueous solution of ammonium sulphate the situation is seen to be further complicated:

$$(NH_4)_2SO_4 \rightleftharpoons 2NH_4^+ + SO_4^{--}$$

The degree of dissociation will be high and there may be reaction of some of the ammonium ions with the solvent viz:

$$NH_4^+ + OH^- \rightleftharpoons NH_4OH \rightleftharpoons NH_3 + H_2O$$

The addition of hydroxyl ions to the solution might be expected to shift the equilibria to the right with

the consequent tendency to evolution of ammonia gas; on the other hand it is surprising that in view of the dilution of alkali employed and of the original ammonium sulphate solution, there whould have been the relatively high losses of ammonia which were observed. An interesting development of this experiment would be to add an equal amount of sodium hydroxide to all beakers to develop pH 8.0 and then rapidly lower the pH of some of the solutions by addition of sulphuric acid. Would the ammonia loss from the solution be dependent upon the alkali initially added or the pH resulting from the addition of the acid?

A second experiment was carried out in which two litre samples of freshly made up ammonium culture solutions at initial pH 6.4 were placed in two litre beakers of cross sectional area 133 sq. cms. The beakers were then placed in the greenhouse and aerated under the normal conditions of an experiment. The data from this experiment are not quoted here since there are so many variables, e.g. the rate of aeration and the greenhouse temperatures. Ammonia loss from the solutions under these conditions was greater and more rapid than in the laboratory experiment, often up to 70% of the dissolved ammonia being lost within forty-eight hours.

EXPERIMENT VIII. THE INFLUENCE OF pH ON GROWTH OF TOMATO PLANTS IN NITRATE AND AMMONIUM CULTURES.

INTRODUCTION:

Such factors as renewal of culture solutions, aeration and pH have been extensively investigated in relation to the growth of higher plants in water culture, using nitrate and ammonium as the source of nitrogen. There appears one clear cut example of the influence of renewal of the culture solution. Pirschle, 1931, found that in static cultures the pH optimum for the growth of maize was 8.0 in ammonium culture and 4.0 in nitrate culture; optimal growth in ammonia was not equal to that in nitrate. Using a flowing culture technique optimal growth occurred at pH 5.6 in both nitrate and ammonium cultures, optimal growth in ammonium culture was slightly greater than in nitrate culture. These results are difficult to explain and do not appear to have been repeated or investigated further. The general conclusion from aeration experiments would appear to be that there is a greater aeration requirement for ammonium cultures than for nitrate, e.g. Clark and Shive, 1934, the effect has not however, been studied in such detail as to be able to give critical oxygen tensions in the culture solution, at which growth differences develop. In this connection it is of interest to note that Spencer, personal communication, 1959, finds a higher DPNH-nitrate reductase activity in root extracts from wheat seedlings

grown in unaerated culture solutions containing nitrate than in root extracts from seedlings grown in similar but aerated solutions. It is suggested that an adaptive nitrate reductase serving as a terminal electron acceptor is formed; the ability to form such an adaptive system might be expected to give a considerable advantage to the nitrate as opposed to ammonia grown plant at low oxygen tensions.

Tiedgens, 1934, working with tomato plants in water culture, reported pH 6 as optimal for growth in nitrate cultures and pH 8 as optimal in ammonium cultures. It is to be noted that in view of the evidence from Experiment I it is unlikely that, under the experimental conditions employed by Tiedgens, the ammonia concentration was equal in his solutions at different pH's. The problem is reinvestigated in the following experiment together with observations on various aspects of the morphology of nitrate and ammonia grown plants.

MATERIALS AND METHODS:

Plants.

Seeds of tomato var. Bonny Best were germinated and the seedlings transferred to culture vessels as described for earlier experiments. The composition of nitrate and ammonium culture solutions are given in Table XXII.

It will be noted that the nitrogen content of the two culture solutions is not equal; this is in order to avoid problems of ammonia toxicity which will be discussed later.

TABLE XXII. COMPOSITION OF NITRATE AND AMMONIUM CULTURE SOLUTIONS.

Culture Solution.	Macronutrients.	M	Micronutrients.	
All-Nitrate Solution.	KNO_3	0.0020M	B	0.100 ppm.
	$Ca(NO_3)_2$	0.0006M	Mn	0.100 ppm.
	KH_2PO_4	0.0004M	Zn	0.010 ppm.
	$MgSO_4$	0.0004M	Cu	0.004 ppm.
	Fe (EDTA complex)	0.6 ppm.	Mo	0.004 ppm.
Ammonium Solution.	KCl	0.0020M	B	0.100 ppm.
	$CaCl_2$	0.0006M	Mn	0.100 ppm.
	$NH_4H_2PO_4$	0.0004M	Zn	0.010 ppm.
	$MgSO_4$	0.0004M	Cu	0.004 ppm.
	Fe (EDTA complex)	0.6 ppm.	Mo	0.004 ppm.

Experimental Design.

Five nitrate cultures were grown and maintained at pH 6.0 by addition of N/50 hydrochloric acid or N/50 sodium hydroxide as required. Ammonium plants were grown at pH 6, 7 and 8, five cultures at each pH, pH were maintained by daily additions of appropriate quantities of N/50 sodium hydroxide following titration of a sample of the culture solution at a glass electrode. Following checks on the nitrogen content of the culture solutions all solutions were changed after ten days and again after 15 days from

the time of planting out. Seventeen days after planting out the plants were harvested, fresh weights measured and samples of the leaves and roots for microscopic examination, placed in a concentrated solution of chloral hydrate (clearing agent).

RESULTS AND DISCUSSION:

The plants grown in ammonium cultures do not differ significantly in fresh weight according to the pH of the solution in which they are grown. After 17 days the shoots, but not the roots, of plants grown in nitrate cultures show a highly significant increase in fresh weight over plants grown in ammonium cultures.

Turner, 1922, showed that for Barley plants in water culture increasing nitrate concentration in the culture solution resulted in a higher ratio of fresh weights of shoots to roots when measured after 49 days of growth. This observation is of interest in relation to the above experiment, though it must be emphasised (c.f. Van der Honert, 1932), that the activities rather than the mass of the root systems is of prime importance physiologically, the two attributes may not be directly related. Thus in Table XXIII the mean fresh weights of the root systems from the four treatments do not differ significantly but it may well be that an enhanced "activity" of the root system of the nitrate grown plant, e.g. in taking up certain essential nutrients, accounts for the subsequent greater growth.

TABLE XXIII. MEAN FRESH WEIGHTS IN GRAMS OF ROOTS AND SHOOTS OF PLANTS AT SEVENTEEN DAYS. EACH VALUE IS THE MEAN FRESH WEIGHT OF 20 PLANTS AFTER SEVENTEEN DAYS.

Culture Solutions.	Fresh weight of shoot gms.	Fresh weight of root gms.	Shoot/Root Ratio.
Nitrate pH 6.5	2.438***	1.260	1.94**
Ammonium pH 6.0	1.538	0.990	1.55
Ammonium pH 7.0	1.646	1.212	1.36
Ammonium pH 8.0	1.482	1.128	1.31.

*** Significant at the 1% level.

** Significant at the 5% level.

Morphologically the root systems do show a difference. After weighing the roots were placed in a strong solution of chloral hydrate clearing agent. After 48 hours roots so treated may be easily split longitudonally and the root hairs on the resulting thin strips, counted. Roots of tomato plants grown in complete or all-nitrate culture solutions are found to possess root hairs along most of their length, but with a marked periodicity in maximum root hair density, which is probably diurnal. Thus under successive microscope fields of 4.0 mms. width moving back along the root from the apex a typical root hair count for half a root, slit longitudonally, would be 0, 216, 12, 0, 16, 224, 20, 8, 20, 380, 76. The roots of plants grown in ammonium cultures possess relatively few root hairs and there is no apparent variation in root hair density along the length of the root; a typical root hair count, made in the manner described would be 0, 6, 8, 23, 16, 5, 7, 30, 33, 8, 16. The root hairs from ammonium grown plants tend to be longer but of approximately the same mean diameter as those from plants receiving nitrate.

There is no significant difference between the number of root hairs on roots from ammonium cultures at different pH's, roots from nitrate cultures however, carry approximately eight times the number of hairs in an equal length of root. This larger number of root hairs is not a consequence of a larger number of

cells per unit of surface area in the nitrate grown root.

TABLE XXIV. TOTAL NUMBERS OF ROOT HAIRS ON TERMINAL 5.0 CMS. OF ROOTS OF SEVENTEEN DAY OLD TOMATO PLANTS. EACH VALUE IS THE MEAN OF TEN ROOTS COUNTED, EACH ROOT BEING TAKEN FROM A SEPARATE PLACE.

Culture Solution.	Number of root hairs.
Nitrate.	2,294 *
Ammonium pH 6.0	388
Ammonium pH 7.0	320
Ammonium pH 8.0	360

* Significant at the 1% level.

It is readily seen that the nitrate grown root will have a much greater surface area than the ammonium grown root, this alone could account for a greater nutrient uptake and consequent growth of the shoot, conversely, as with fresh weight, the root hair number may not reflect the "activity" of the root system. The periodicity in the production of root hairs by nitrate grown roots probably corresponds to a similar periodicity in translocation of products from the shoot, such as is indicated by the work of Sachs, 1892, Mason and Maskell, 1928, Loomis, 1933, and Goodall, 1946; this being so a diminished shoot

metabolism, and in particular photosynthesis, is suggested in the ammonia grown plant. It must finally be noted that the conditions of a culture solution are very different from those of a soil, thus some species are found to form root hairs when grown in soil but not in water cultures, the phenomenon here described may well represent a state of affairs peculiar to roots grown in water cultures.

EXPERIMENT IX. THE EFFECT OF AMMONIA CONCENTRATION ON PLANT GROWTH.

INTRODUCTION:

Before pursuing further this suggestion of an effect on the metabolism of the photosynthetic tissues it is necessary to meet certain other problems in the comparison of growth on nitrate and ammonium. In particular the possible toxicity of the concentrations of ammonia used in foregoing experiments might account for the reduced growth in ammonium cultures; if such a toxicity does exist it is perhaps surprising that the initial effect on fresh weight is not more marked in the root system, which is in contact with the culture solution. As mentioned in connection with the previous experiment however, an effect on root "activity" may not be immediately reflected in a more readily measured property of the root such as fresh weight, dry

weight, root length or root hair numbers. Arising out of the work of Prianishnikov, 1922 et seq., on the metabolism of seedlings, was the idea of ammonia being toxic to plant tissues and of amide formation as a detoxification mechanism. This may hold true for high or "unphysiological" concentrations of ammonia but the concept is not supported by the work of Vickery et al, 1938, 1939, on rhubarb leaves, or of Burkhart, 1938, using etiolated seedlings; working with ammonia concentrations probably nearer those to which the plant is subjected when growing under "natural" conditions. Pirschle, 1929, followed the Prianishnikov concept to the conclusion that in order to obtain comparable growth of plants using nitrate or ammonium nitrogen it is necessary to supply only as much ammonia to a culture as is equal to that made available by the reduction of nitrate in the cells of a plant growing in a nitrate culture. Exactly this state of affairs is difficult to achieve in practice since the rate of growth of plants in the greenhouse varies from day to day and more particularly from one experiment to the next according to the prevailing weather. In consequence a curve of plant nitrogen content plotted against time, obtained from one experiment can not be used to indicate the necessary nitrogen level to be supplied in a subsequent experiment where the growth rate may be different. A nearer approximation can be obtained by knowing the

change in percentage nitrogen content of the plant with increasing fresh weight and supplying amounts of nitrogen calculated from the day to day changes in fresh weight of the plants. The main objection to this proceedure is the number of plants which must be grown and the consequent labour involved. In the following experiment analysis of the residual nitrogen content of the culture solution is taken as an index of requirement, this measurement though convenient suffers from the difficulty of applying a correction for the rate of ammonia loss to the atmosphere. A further objection to this measurement is that the nitrogen taken up into the plant may not be immediately metabolised, and it is possible that ammonia enters the plant more rapidly than nitrate, Trelease and Trelease, 1935, thus measurement of residual nitrogen in ammonium cultures will suggest a further nitrogen requirement although in fact, the ammonia taken up has not yet been metabolised. Again, it may be that the plant can tolerate a higher level of free nitrate than of free ammonium nitrogen. Difficulties such as those of ammonia loss and bacterial growth could probably be overcome by use of a "flowing culture" technique, this proceeduze has not however, been tried.

MATERIALS AND METHODS:

Plants.

Seedlings of tomato var. Bonny Best were raised in the manner described for previous experiments.

TABLE XXV. INITIAL COMPOSITION OF NITRATE AND AMMONIUM CULTURE SOLUTIONS.

Culture Solution.	Macronutrients.		Micronutrients.	
Culture 1.	KNO_3	0.0020 M	B	0.100 ppm.
All Nitrate	$Ca(NO_3)_2$	0.0006M	Mn	0.100 ppm.
Solution.	KH_2PO_4	0.0004 M	Zn	0.010 ppm.
	$MgSO_4$	0.0004 M	Cu	0.004 ppm.
	Fe (EDTA complex)	0.6 ppm.	Mo	0.004 ppm.
Culture 2.	KNO_3	0.0020 M		
Complete	$Ca(NO_3)_2$	0.0006 M	As for	
Culture	$(NH_4)H_2PO_4$	0.0004 M	Culture 1.	
Solution.	$MgSO_4$	0.0004 M		
	Fe (EDTA complex)	0.6 ppm.		
Culture 3.	KCl	0.0020 M		
Ammonium	$CaCl_2$	0.0006 M	As for	
Culture	$(NH_4)H_2PO_4$	0.0004 M	Culture 1.	
Solution.	$MgSO_4$	0.0004 M		
	Fe (EDTA complex)	0.6 ppm.		
Culture 4.	As for Culture 3		As for	
Ammonium	except:		Culture 1.	
Culture Soln.	$(NH_4)H_2PO_4$	0.0004M		

Culture 5.	As for Culture 3 except:	As for Culture 1.
	$(NH_4)H_2PO_4$ 0.00004 M	
	KH_2PO_4 0.00036 M	

Culture Solutions.

Five culture solutions were employed, the composition of each is given in Table XXV.

Six replicate culture vessels of each solution were set up and corrected to an initial pH of 6.0. The pH of all solutions was maintained at 6.0 by daily additions of appropriate quantities of N/50 hydrochloric acid or N/50 sodium hydroxide. At two day intervals the ammonia content of each culture solution was determined and, where necessary, ammonium chloride added to bring the ammonia level to the initial value. At 8, 12, 16 and 20 days from the time of setting up the cultures, one plant was harvested from each vessel. The plants were oven dried in a current of air at 80° C. for five hours and then placed in a vacuum desiccator over phosphorus pentoxide for forty-eight hours before being weighed.

RESULTS:

The effect of varying concentrations of ammonia on the dry weight production of the plants is shown in Table XXVI.

Plants receiving nitrate, culture solutions 1 and 2, were larger and of a lighter green colour than ammonia grown plants. There is no significant difference between the dry weights of ammonia grown plants according to the dilution of the ammonia, plants receiving both nitrate (2×10^{-3}M) and ammonia (4×10^{-4}M) however, were

TABLE XXVI: EFFECT OF AMMONIA CONCENTRATION ON GROWTH OF TOMATO PLANTS IN CULTURE SOLUTION AT pH 6. GROWTH RECORDED AS DRY WEIGHT OF WHOLE PLANT IN GRAMS. EACH VALUE IS THE MEAN DRY WEIGHT OF SIX PLANTS.

Composition of Culture Solution.	Age of plant at time of harvest.			
	8 days.	12 days.	16 days.	20 days.
Culture 1. All Nitrate.	0.043	0.111	0.422	1.031
Culture 2. Nitrate and Ammonia	0.040	0.127	0.410	1.002
Culture 3. Ammonia .	0.041	0.081	0.220	0.336
Culture 4. Ammonia.	0.039	0.086	0.200	0.398
Culture 5. Ammonia.	0.041	0.104	0.218	0.401.

similar in appearance and dry weight to plants receiving nitrate alone. Thus it is concluded that reduced growth on ammonia is associated with absence of nitrate rather than toxicity of ammonia unless, as would seem less likely, nitrate affects the metabolism of the plant in such a way as to afford a "protection" against the deleterious effects of ammonia.

In connection with this experiment analyses of total amide were made on some plants, plants were not chosen at random and analyses were not statistically replicated, but there was a clear indication of a high amide concentration (expressed in terms of fresh weight) in plants receiving ammonia at 4×10^{-3}M, in plants receiving ammonia at 4×10^{-4} to 4×10^{-5}M; on the other hand the amide content was similar to that of plants grown on all nitrate and on complete culture solutions.

The observations on root hairs noted in Experiment 7 suggested the possibility of an impeded flow of appropriate metabolites from shoot to root. This possibility leads to the suggestion that the "ammonia plant" may in fact be the result of a translocation blockage or impediment, so that the reduced growth of the ammonia plant might be explained in terms of a limiting rate of ammonia translocation from root to shoot. Against this the data from Experiment 5 suggests that free ammonia readily enters the plant and is probably translocated to the shoot.

Oland and Yemm, 1956, found arginine constituted 60% of the soluble nitrogen extractable in 70% ethanol from apple stems and Bollard, 1956, found glutamine and asparagine to be major components of the soluble nitrogenous fraction of the xylem sap of several woody plants. If from this type of data one accepts the conclusion that arginine and amides probably represent a large proportion of the translocated nitrogen of the plant then it might be argued that since these products are known to be readily formed from ammonia, arginine via the arginine cycle, glutamine by glutamine synthetase, asparagine with less certainty; then the ammonia grown plant should have access to an easily translocatable form of nitrogen. It must be noted however, that these observations are made mainly on woody plants, and as noted in the introductory discussion there is a strong suggestion that woody plants differ from herbaceous plants in regard to the localisation of nitrate and ammonia assimilation.

It might be supposed that an experiment using nitrate and ammonia labelled with N^{15} could decide the question of the relative rates of translocation of the two forms of nitrogen. The difficulties associated with such a comparative experiment are however, considerable; they may be classified broadly into two groups:-

(1) problems associated with deciding the optimal range of conditions of pH, concentration, temperature,

aeration and proportionality with other ions, for the supply of nitrate and ammonium ions;

(2) problems associated with the condition of the plant at the time of commencing an experiment; thus it may be that the rate of translocation of nitrate or ammonia from root to shoot is influenced by the amounts of nitrate or ammonia already present in the shoot. It is difficult to decide how these factors might be manipulated to determine optimal conditions for a comparative experiment; techniques such as nitrogen starvation of the plant immediately prior to feeding $N^{15}H_4$ and $N^{15}O_3$ are unsatisfactory since other aspects of metabolism having secondary effects on translocation are likely to be affected.

EXPERIMENT X. THE LIGNIN AND STARCH CONTENTS OF NITRATE AND AMMONIA GROWN PLANTS.

INTRODUCTION:

In earlier experiments involving the grinding up of plants, it was frequently noted that ammonia grown plants are more easily ground than nitrate grown plants. A simple histochemical testing of tissue sections with aniline sulphate showed a considerably stronger lignin reaction in nitrate than in ammonia grown tissues. In view of the suspected influence on carbohydrate metabolism in ammonia grown plants, it was decided to estimate the

lignin content of nitrate and ammonia grown plants and to see if the softness of the latter could in fact be correlated with a reduced lignin content. Observations on stomatal index of the plants are also included here.

MATERIALS AND METHODS:

Plants.

Tomato plants var. Bonny Best were grown under the conditions described for earlier experiments.

Culture Solutions.

Culture solutions of the composition described for Experiment IX, Culture 2 (Complete) and Culture 4 (Ammonia 4 x 10^{-4}M), were prepared.

Experimental Design.

The replicate culture vessels of each culture solution were set up and corrected to an initial pH of 6.0. The pH's of all cultures were maintained at 6.0 by daily additions of appropriate quantities of hydrochloric acid or N/50 sodium hydroxide. At two day intervals the ammonia content of each culture solution was determined and where necessary ammonium chloride added to bring the ammonia level to the initial value. At 24 days from the time of setting up the cultures each plant was harvested, roots and shoots separated, fresh weights measured, and analyses of lignin content made.

Analyses.

The estimation of lignin presents many difficul-

ties and several methods have been tried for different types of tissue requiring different extraction proceedures. The proceedure adopted is essentially Siegal's, 1953, modification of the method of Norman, 1937, for soft tissues. The separated roots and shoots were extracted successively with boiling water, hot 75% ethyl alcohol, hot 95% ethyl alcohol, and an ethyl alcohol - petroleum - ether mixture. The residue from these extractions was dried and then treated with 72% sulphuric acid at 2° C for twelve hours. The acid was diluted to 3% and the residue hydrolysed by boiling for one hour, the residue (lignin) was then separated by centrifuging for ten minutes at 300g, dried and weighed.

RESULTS:

The difference in growth and in lignin content between plants receiving nitrate and ammonium, and plants receiving only ammonium nitrogen, is clearly seen, and it is of interest to note that the greatest differences after twenty four days, are in the shoots. If, as seems possible, the biosynthesis of lignin proceeds initially via the cyclisation of a seven carbon unit such as sedoheptulose to form shikimic acid, then it might be said that this data provides further evidence for an effect on the photosynthetic system in the shoot. There is however, a basic weakness in this method of experiment. By the twenty-fourth day plants receiving nitrate are larger and have

TABLE XXVII. THE FRESH WEIGHTS IN GRAMS OF ROOTS AND SHOOTS OF COMPLETE AND AMMONIA CULTURE GROWN PLANTS AT 24 DAYS. EACH VALUE IS THE MEAN FRESH WEIGHT OF TWELVE PLANTS.

Culture Solution.	Mean Fresh Weight of Shoot. gms.	Mean Fresh Weight of Root. gms.
Complete culture	8.41	2.14
Ammonium Culture	2.43	1.11

TABLE XXVIII. THE WEIGHT AND PERCENTAGE OF FRESH WEIGHT OF LIGNIN IN SHOOTS AND ROOTS OF COMPLETE CULTURE AND AMMONIA CULTURE GROWN PLANTS AT 24 DAYS. EACH VALUE IS THE MEAN OF SIX ANALYSES.

Culture Solution.	Weight of Lignin. per shoot mgms.	Lignin content. as % of F.W.	Weight of Lignin. per roots mgms.	Lignin Content as % of F.W.
Complete	124.5	1.48	43.0	2.01
Ammonium	8.0	0.33	11.9	1.07

more leaves than ammonia grown plants, so that some of their tissues are older and more particularly of a different "physiological age" from the tissues of ammonia grown plants. It is well known that the rate of lignification increases with age in many plants and with increasing physiological age in particular tissues; the data in Table XXVIII may simply reflect this state of affairs.

The time needed for analysis and variability in results make it difficult to avoid this objection with lignin data; in the following experiments however, an analysis of regression examining the change in level of a particular constituent in relation to the extent of growth is used to meet this difficulty.

Measurement of stomatal indices revealed no differences between nitrate and ammonia grown plants; values in both cases were 2·6

Of further interest in this experiment was the observation that when leaves from nitrate grown plants are homogenised and subsequently centrifuged, the sediment contains a prominent white layer which is found to consist largely of starch grains, leaves from ammonia grown plants treated in this way yield hardly any starch.

EXPERIMENT XI. THE UPTAKE OF PHOSPHORUS INTO THE ORGANIC AND INORGANIC FRACTIONS OF THE PLANT.

INTRODUCTION:

Petrow, 1917, found that plants grown in darkness and supplied with sugars grew more rapidly when ammonia formed the source of nitrogen, than when nitrate was the form of the nitrogen supplied, in light however, growth was more rapid on nitrate. Related to this finding is the observation made earlier in the present work, that at normal light intensities there is a greater growth of nitrate as compared to ammonia grown plants, whereas at low light intensities such differences in growth are less pronounced. Doman and Vaklinova, 1938, found lower levels of carbohydrates in ammonia than in nitrate grown plants. These observations coupled with the finding in the present experiments of a lower starch and lignin content, and the failure of the diurnal periodicity of root hair formation in ammonia grown plants tends to suggest that photosynthesis is in someway limited in ammonia as compared to nitrate grown plants. Arnon, 1939, examined and compared the composition of barley plants grown in water cultures containing nitrate or ammonium nitrogen; one notable difference in the composition of the material was a higher total phosphorus content in ammonium than in nitrate grown plants. If barley responds to nitrate and ammonia in a manner simi-

ler to tomato plants this result might be regarded as surprising since if the rate of photosynthesis is slower in the ammonia grown plant and hence less carbohydrate is formed, one might expect a reduction in the amounts of phosphorus required for such reactions as the formation of sugar phosphates and their derivatives. Again the very high ratio chloroplast mass to mitochondrial mass in green leaves, of the order 6 : 1 on a protein basis, and the relatively high rates of photosynthetic phosphorylation obtained with isolated chloroplasts, e.g. Avron et al, 1958, suggests that a large proportion of the inorganic phosphate esterified in the leaf in the course of metabolism represents photosynthetic rather than oxidative phosphorylation. This being so a reduction in photosynthesis might be expected to be associated with a reduction in photosynthetic phosphorylation and hence a lower phosphate requirement in the shoot. Against this it might be argued that there is a greater need for phosphate as a buffer in the sap of the ammonia grown plant, a mechanism for this type of effect is at the present state of knowledge difficult to find and prove. These speculations resulted in the following investigation of the phosphorus uptake by nitrate and ammonia grown plants and in particular an examination of the form in which the phosphate is present in the plant.

MATERIALS AND METHODS:

Plants.

Seeds of tomato variety Bonny Best were germinated

and the seedlings transferred to water cultures as described for earlier experiments.

Culture Solutions.

A complete culture solution was made up as in Table XXV, Culture 2, and an ammonium culture solution as in Table XXV, Culture 4. To compensate for ammonia uptake and loss to the atmosphere, 0.0428 gms. of ammonium chloride was added to each culture on the seventh and thirteenth days, following measurements of the ammonia level in the solutions.

The pH of the ammonium culture was maintained by appropriate additions of N/50 sodium hydroxide, following titration of a sample of the culture solution at a glass electrode.

Experimental Design.

Eighteen ammonia and eighteen Complete cultures were grown, six cultures (24 plants) of each treatment being harvested on each of three dates 8, 13 and 17 days from the time of placing the plants in the culture vessels. Of the twenty four plants from one treatment at each harvest, twelve were used for fresh weight, dry weight, and total phosphorus measurements, and the remaining twelve for fresh weight and inorganic phosphorus estimations. Roots and shoots were weighed and analysed separately.

Analyses.

Total phosphorus :- the dried material was digested with 1.0 ml. concentrated sulphuric acid, 1.0 ml.

72% perchloric acid and 5.0 ml. concentrated nitric acid in a microkjeldahl flask. After dilution with distilled water, orthophosphate was estimated by the method of Strickland et al, 1956.

Inorganic phosphate:- fresh plant material was ground in a pestle and mortar with a known volume of ice-cold 3% trichloracetic acid, approximately 50 mls. of acid per gram fresh weight of tissue. The homogenate was centrifuged at 500xg and 0° C. for five minutes and 1.0 ml. aliquots of the supernatant analysed for orthophosphate, according to the method of Strickland et al, 1956. The analytical proceedure of Strickland et al was originally developed for the estimation of acid phosphatase activity in brain tissues and is shown to avoid hydrolysis of labile phosphate esters.

Organic phosphate values are derived by subtraction of the inorganic phosphate from the total phosphate present in the plants.

RESULTS:

The particular enhancement of shoot growth in nitrate grown plants already noted in Experiment VII is more clearly seen in the fresh and dry weight data of Tables XXIX and XXX, and figure 11.

The ratio of fresh and dry weights of both roots and shoots is not significantly different between balanced and ammonium cultures at any stage of the experiment.

TABLE XXIX: FRESH WEIGHT IN GMS. OF NITRATE AND AMMONIA GROWN PLANTS ON WHICH INORGANIC PHOSPHATE ESTIMATIONS WERE SUBSEQUENTLY CARRIED OUT.

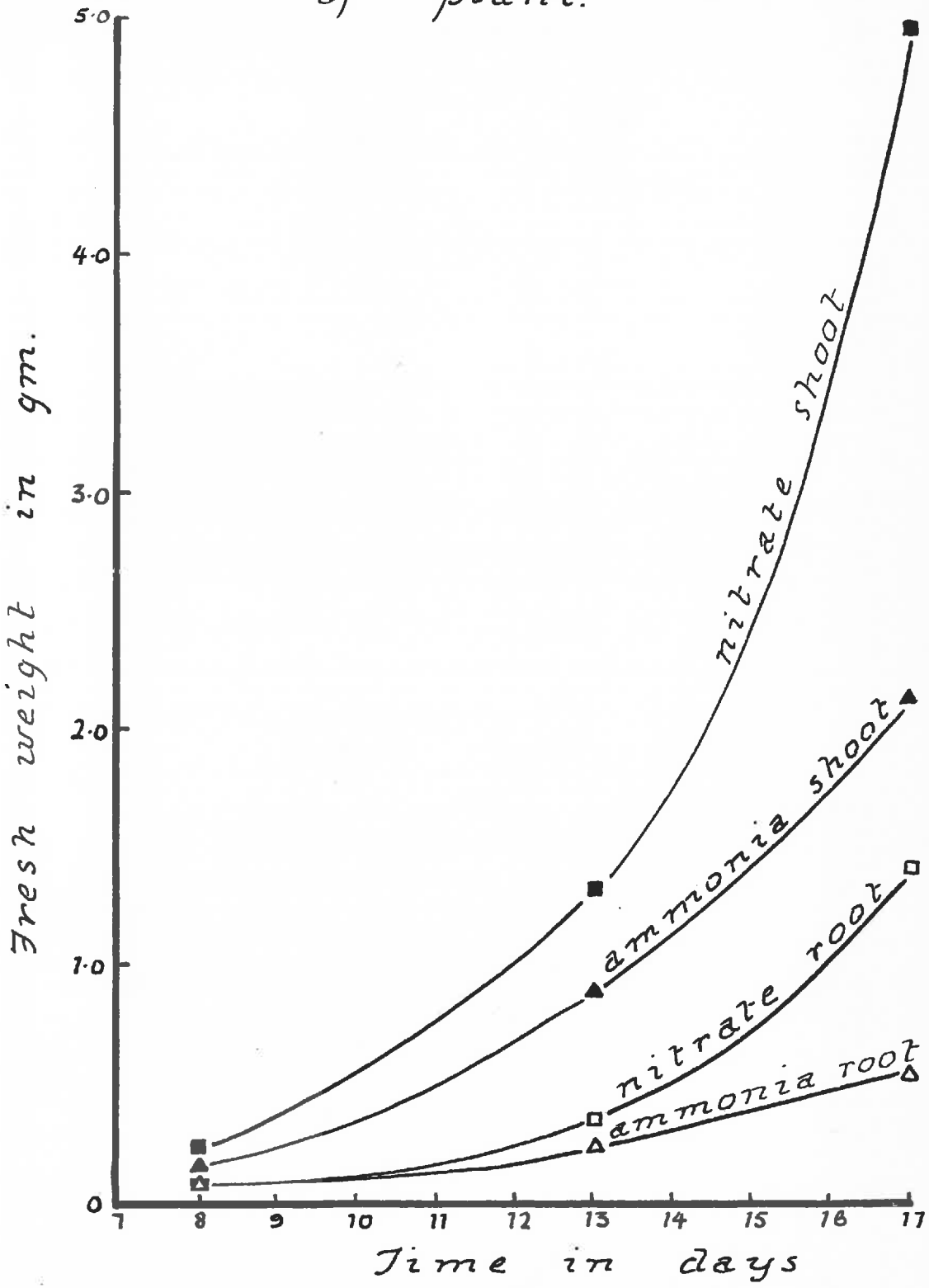
EACH VALUE IS THE MEAN FRESH WEIGHT OF TWELVE PLANTS.

Day of Harvest.	Complete Cultures. Fresh Weight. of shoot gms.	Fresh Weight. of root gms.	Ratio. shoot : root.	Ammonium Cultures. Fresh Weight. of shoot gms.	Fresh Weight. of root gms.	Ratio shoot : root.
8th	0.224	0.052	4.31	0.187	0.057	3.28
13th	1.316	0.345	3.82	0.883	0.273	3.23
17th	4.948	1.471	3.36	2.150	0.483	4.45

TABLE XXX: DRY WEIGHT IN GRAMS OF NITRATE AND AMMONIA GROWN PLANTS ON WHICH TOTAL PHOSPHATE ESTIMATIONS WERE SUBSEQUENTLY CARRIED OUT. EACH VALUE IS THE MEAN DRY WEIGHT OF TWELVE PLANTS.

Day of Harvest.	Complete Cultures. Dry Weight. of shoot gms.	Dry Weight. of root gms.	Ratio shoot : root.	Ammonium Cultures. Dry Weight of shoot gms.	Dry Weight of root gms.	Ratio shoot : root
8	0.0164	0.0032	5.13	0.0108	0.0021	5.14
13	0.0953	0.0149	6.40	0.0626	0.0114	5.49
17	0.4114	0.0634	6.49	0.1783	0.0303	5.89

Figure 11 — Fresh weight plotted against age of plant.



As found by Turner, 1922, the ratio of shoot weight to root weight tends to increase as the plant grows, Table XXX, but as noted in an earlier experiment, the increase in this ratio is more rapid in the nitrate than in the ammonia grown plant.

By the time of the second harvest, thirteen days, the mean fresh and dry weights of both shoots and roots differ significantly at the 0.1% level between balanced and ammonium cultures.

To express the total and inorganic phosphate content of the plant as a "concentration" introduces the "condition of the plant" factor into the data which might be misleading. For this reason the phosphate data in Tables XXXI and XXXII is quoted as mean phosphate content in micrograms per shoot, and the data subsequently examined in terms of the regression of phosphate content on plant weight.

The regression of total phosphate content of the shoot on dry weight of the shoot for each treatment at the 17 day harvest is plotted in figure 12.

Considering these two lines for the regression of total phosphate content on dry weight at the harvest on the seventeenth day:-

For shoots grown in complete culture the regression coefficient (b) of total phosphate on dry weight = 5918.9

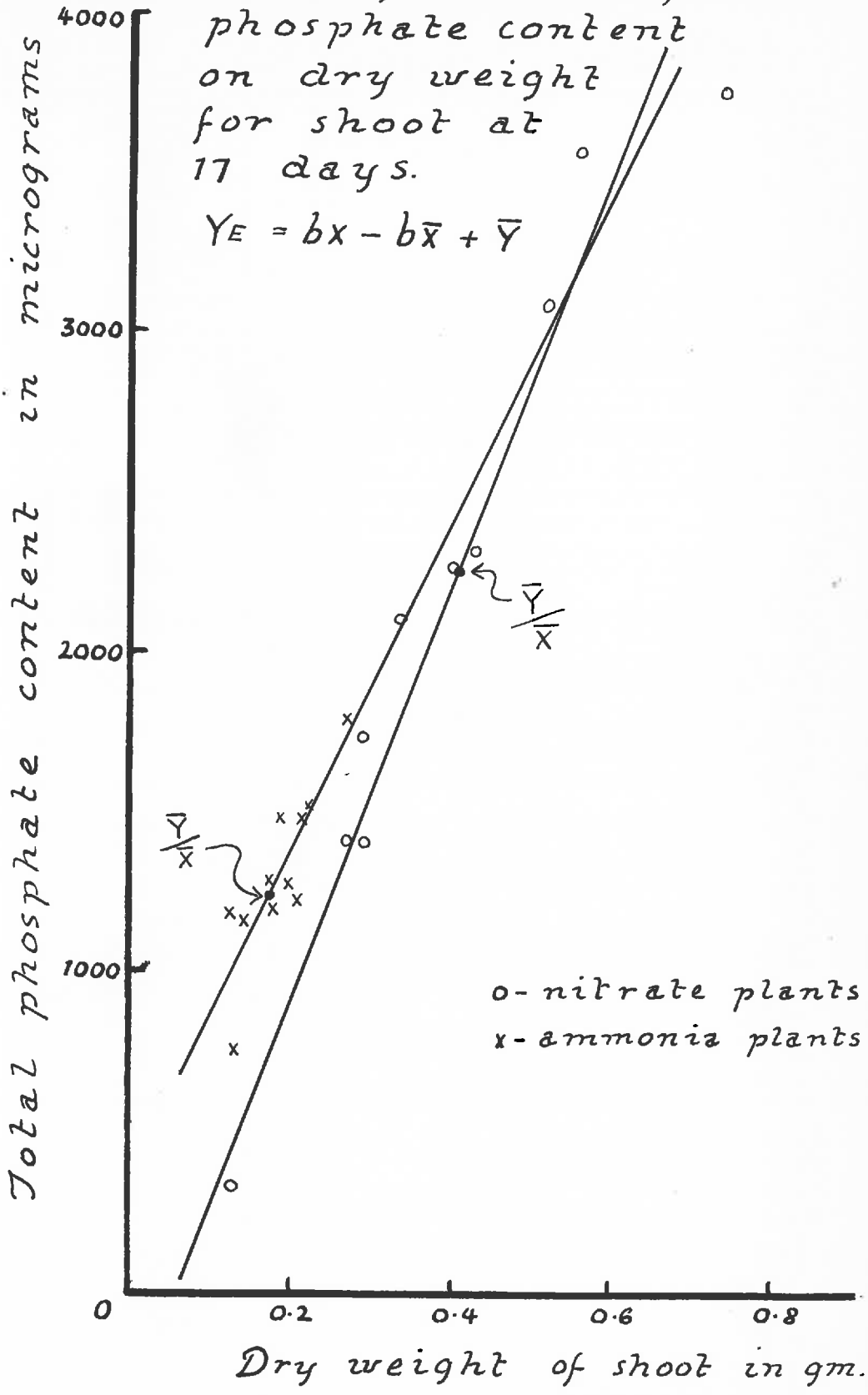
This regression coefficient is significant at 0.1%, $t = 6.361$, $n = 10$.

TABLE XXXI: TOTAL PHOSPHATE CONTENT OF SHOOTS AND ROOTS EXPRESSED AS MICROGRAMS PER SHOOT, AND PER ROOT. EACH VALUE REPRESENTS THE MEAN OF TWELVE ANALYSES.

Day of Harvest.	Complete Cultures. Total phosphate. per shoot.	Complete Cultures. Total phosphate. per root.	Ammonium cultures. Total phoaphate. per shoot.	Ammonium cultures. Total phoaphate per root.
8	196.1	59.6	105.0	33.9
13	836.9	200.33	578.0	208.7
17	2327.3	649.2	1266.7	376.7

Figure 12– Regression of total phosphate content on dry weight for shoot at 17 days.

$$Y_E = bx - b\bar{x} + \bar{Y}$$



For shoots grown in ammonium culture the regression coefficient (b') of total phosphate on dry weight = 5216.3

This regression coefficient is significant at 0.1%, t = 10.020, n = 10.

Following R.A. Fisher's analysis (171) for the comparison of regression coefficients one has

$$t = \frac{b - b'}{\text{Standard Error of } b - b'} = 0.3157 \qquad n = 20.$$

Thus the slopes of the two regression lines do not differ significantly. An analysis of variance on the other hand shows the means of the two treatments are very different:-

$$F = t^2 = 175.6 \qquad N_1 = 1 \qquad N_2 = 21$$

This analysis is confirmed by inspection of figure 12, which indicates that the similarity of regression but difference between the means of the two lines can be accounted for by the data from the two treatments occupying different regions of a common regression line.

The regression of inorganic phosphate content on fresh weight is plotted in figure 13.

Analysis of regression of inorganic phosphate content on fresh weight, was carried out:-

Considering the two lines, figure 13, for the regression of inorganic phosphate content on fresh weight at the harvest on the seventeenth day:-

For shoots grown in complete culture, the regression coefficient (b) of inorganic phosphate on fresh weight = 114.94.

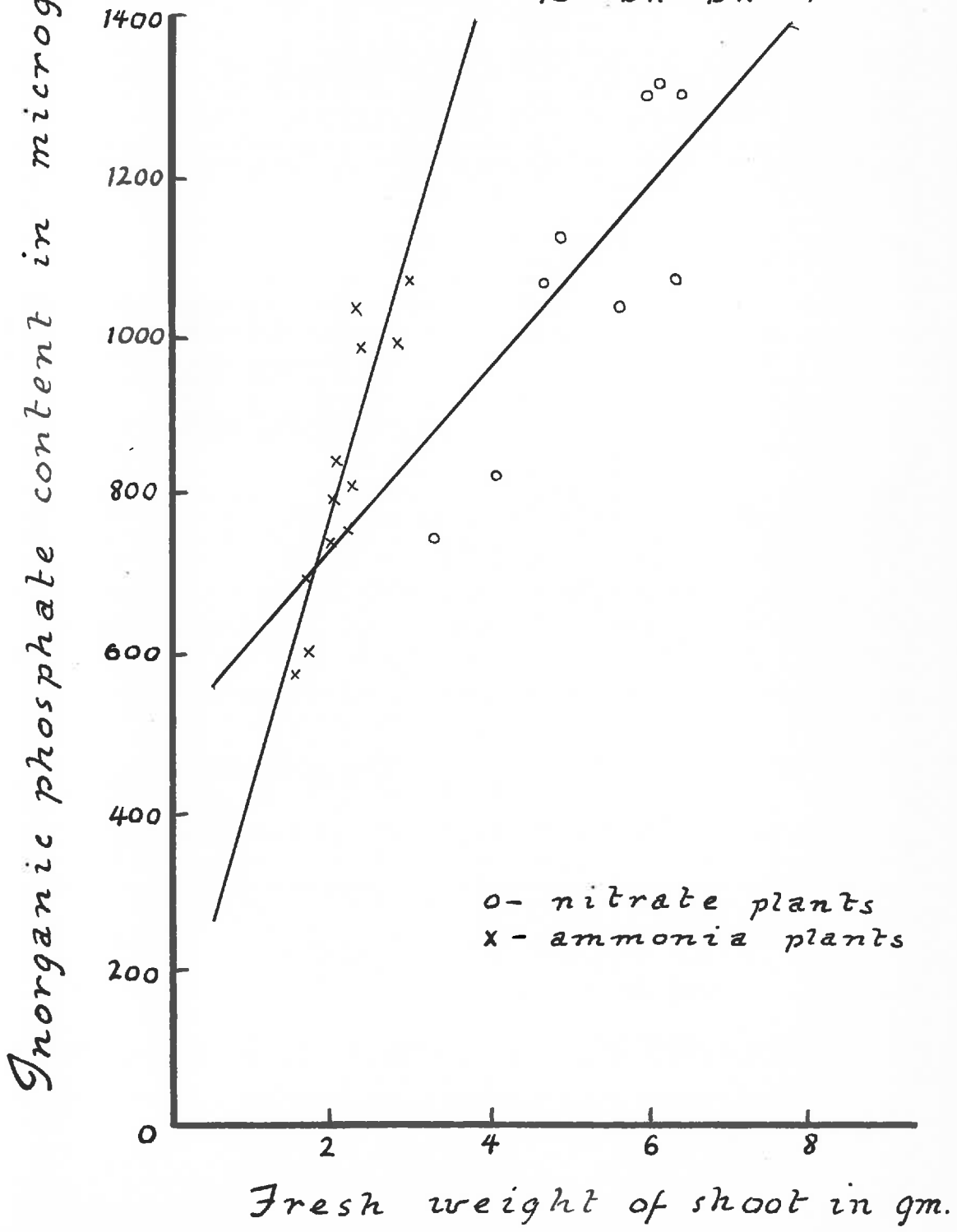
This regression coefficient is significant at

TABLE XXXII. THE INORGANIC PHOSPHATE CONTENT OF SHOOTS AND ROOTS EXPRESSED AS MICROGRAMS PER SHOOT OR PER ROOT. EACH VALUE REPRESENTS THE MEAN OF TWELVE ANALYSES.

Day of Harvest.	Complete Cultures. Inorganic phosphate per shoot.	Complete Cultures. Inorganic phosphate per root.	Ammonium Cultures. Inorganic phosphate per shoot.	Ammonium Cultures. Inorganic phosphate per root.
8	124.9	27.2	121.4	41.3
13	375.3	81.8	400.0	82.3
17	1037.5	305.7	627.0	247.8

Figure 13 – Regression of inorganic phosphate on fresh weight of shoot at 17 days.

$Y_E = bX - b\bar{x} + \bar{Y}$



5% t = 2.497 n = 9.

For shoots of plants grown in ammonium culture the regression coefficient (b') of inorganic phosphate on fresh weight = 352.78.

This regression coefficient is significant at 0.1%

t = 6.97 n = 10.

Comparing the regression lines

$$t = \frac{b - b'}{\text{Standard Error of } b - b'}$$

t = 2.15 n = 17. Significant at 5% level.

Thus the inorganic phosphate content of the shoots of ammonia grown plants rises to a greater level in relation to fresh or dry weight than is the case with shoots from plants grown on complete cultures. Since the total phosphate content of the shoot does not show a similar regression then it follows that this increase in inorganic phosphate must be derived from the organic phosphate in the tissue. Values for the organic phosphate content of the plants, obtained by subtracting inorganic from total phosphate figures, are given in Table XXXIII, along with the ratio of inorganic to organic phosphate (Pi/Po).

The ratio Pi/Po in Table XXXIII gives further indication of the tendency to inorganic phosphate increase in comparison with organic phosphate in the tissues of ammonia grown plants.

A similar analysis of shoot data at 13 days re-

TABLE XXXIII. THE ORGANIC PHOSPHATE CONTENT (Po) OF SHOOTS AND ROOTS EXPRESSED AS MICROGRAMS PER SHOOT OR ROOT. EACH VALUE IS OBTAINED AS THE DIFFERENCE BETWEEN THE MEAN AND TOTAL PHOSPHATE CONTENT OF TWELVE PLANTS AND THE MEAN INORGANIC PHOSPHATE CONTENT OF TWELVE PLANTS.

Day of Harvest.	Complete cultures. Shoot. Po per shoot.	Ratio Pi/Po.	Root. Po per root.	Ratio Pi/Po.	Ammonium cultures. Shoot. Po per shoot.	Ratio Pi/Po	Root. Po per root	Ratio Pi/Po
8	71.2	1·75	32.4	0·84	-	-	-	-
13	461.6	0.81	118.5	0·70	178.0	2.25	126.4	0·65
17	1289.8	0.80	343.5	0·89	439.7	1.88	128.9	1·91

veals an even earlier onset of the phosphate unbalance:-
The regression coefficient (b) of total phosphate on dry weight for shoots of nitrate grown plants = 7941.6
The regression coefficient (b') of total phosphate on dry weight for shoots of ammonia grown plants = 8092.7

These two regression coefficients do not differ significantly. The regression coefficient (b) of inorganic phosphate on fresh weight for the shoots of nitrate grown plants = 155.0
The regression coefficient (b') of inorganic phosphate on fresh weight for the roots of ammonia grown plants = 369.1

These two regression coefficients are significantly different at the 1% level. The argument thus holds as stated in the case of the seventeenth day harvest, inorganic phosphate content is increasing more in relation to fresh weight than is organic phosphate content, in the shoots of ammonia grown plants.

Roots: An examination of the data obtained from the analysis of roots for total and inorganic phosphate content reveals an interesting situation:-

Analysis of roots at 17 days.

The regression of total phosphate content on dry weight does not differ significantly between nitrate and ammonia grown roots.
The regression coefficient (b) of inorganic phosphate content on fresh weight for nitrate grown roots = 214.0
The regression coefficient (b') of inorganic phosphate content on fresh weight for ammonia grown roots = 393.5

These two regression coefficients differ significantly at 1% $t = 4.19$ $n = 20$. Thus, as was the case with the shoot, the ammonia grown root at 17 days shows a greater increase of inorganic phosphate with increasing fresh weight than does the nitrate grown root.

Turning now to the analysis of roots at 13 days:-
The regression of total phosphate content on dry weight does not differ significantly between nitrate and ammonia grown roots.
The regression coefficient (b) of inorganic phosphate content on fresh weight for nitrate grown roots = 238.3
The regression coefficient (b') for inorganic phosphate content on fresh weight for ammonia grown roots = 263.59

These two regression coefficients do not differ significantly. Thus at the 13 day harvest the inorganic phosphate content of the shoot but not of the root of ammonia grown plants is increasing significantly more in relation to fresh weight than is the case in nitrate grown plants. Therefore if this change in the phosphate metabolism in ammonia grown plants is in any way a primary effect then it would seem likely that it is located in the shoot rather than in the root, or the effect may be on reactions which proceed more rapidly in the shoot than in the root so that the effect becomes apparent first in the shoot tissues. Chapter III is therefore concerned with an investigation of the effect of nitrate and ammonium ions on the phosphorus metabolism of the shoot.

CHAPTER III.

Experiment XI revealed, on analysis of regression, that the "level of inorganic phosphate" increased more with increasing fresh weight in the shoots of plants grown on ammonia than in the shoots of plants grown on nitrate, and that this inorganic phosphate was derived from organic phosphate rather than being a consequence of an enhanced uptake of inorganic phosphates.

The phrase "level of inorganic phosphate" is used to refer to the total amount of inorganic phosphate obtained from a particular organ or tissue, when extracted under certain conditions. This type of procedure does not take account of the possible heterogeneity, with respect to morphology, age or function, of the tissue or organ analysed. Towards a more detailed locating of particular reactions within cells, tissues and organs of plants many techniques have been tried, their applicability in a given instance depending on the type of material, and the nature of the reaction under investigation.

One method, the dissection of a tissue into smaller, if possible morphologically uniform, pieces, and analysis of these fragments, may reveal the presence of a particular system in one type of cell. A disadvantage of such a method is that dissection of the tissue almost always results in a modification of the cell environment and hence may tend to alter the reactions going on within the cells.

Similar difficulties arise with histochemical techniques whereby whole organs or tissue slices are perfused with substances acting as indicators for particular reactions.

The formation and esterification of inorganic phosphate is a particularly complex problem, since there are probably a large number of different pathways in the living organism involving the transformation of phosphate between the inorganic and organic forms. These reactions may be located in different cells or at various sites within the same cell, and their individual rates may be differently affected by particular changes in the cell environment. Again a particular reaction may be operative during only a part of the functional life of the cell, in such a case, analysis of a group of cells of varying ages will give only an average value for the whole group. Given the possibility of isolating different types of cells and if the different inorganic phosphate forming and inorganic phosphate esterifying reactions were few and separately located in the different types of cell, then they might be investigated by inhibitor and kinetic studies. Even allowing for technical difficulties of measurement, such experiments will yet be inadequate where a series of different reactions all proceeding within one type of cell, contribute to the total turnover of phosphate.

The site of a particular reaction may be further defined by separation of the different cell organelles and investigation of their metabolism in vitro. One great diffi-

culty with such studies is that they are still further removed from the conditions in the living organism; thus for example, whilst it may be possible to determine the optimal conditions for the formation or esterification of inorganic phosphate by isolated mitochondria, there is no certainty that such optimal conditions ever occur within the cell, and if they did there still remains the possibility of their functioning having been influenced by the proximity of adjacent organelles within the intact cell. If the processes involved in the turnover of phosphate in the cell are enzymic reactions, it may be possible to isolate the enzymes concerned. Perhaps the main value of such studies is in finding out what biochemical reactions might be involved in the turnover of phosphate, but to relate the structure and functioning of an isolated enzyme to its structure and functioning in the living cell, breaches one of the major problems of biochemistry at the present time.

In this chapter, Experiment 13 is an examination of the nitrate-stimulated esterification of phosphate in relation to the age of the leaves in which it occurs, and Experiment 14 is concerned with an investigation of one particular group of isolated organelles, the chloroplasts, in relation to their functioning in the formation of organic phosphate.

EXPERIMENT XII. THE EFFECT OF LIGHT ON THE ESTERIFICATION OF PHOSPHATE IN THE SHOOTS OF AMMONIA GROWN PLANTS FOLLOWING THE FEEDING OF NITRATE.

INTRODUCTION:

Kandler, 1955, using inhibitors on intact cells of Chlorella provided strong evidence for a phosphorylation reaction, unique to photosynthesis, and independent of respiratory chain-type oxidative phosphorylation. Following the general hypothesis of Ruben, 1943, this so-called photosynthetic phosphorylation was demonstrated by Arnon et al, 1955, using isolated chloroplasts of spinach. This phosphorylation was demonstrable in both whole chloroplasts and chloroplast fragments and was found to be an anaerobic process dependant upon light. As noted in the introductory discussion, experiments by several workers, e.g. Burstrom, 1942, Rombeck, 1943, and Stoy, 1955, suggest that nitrate assimilation in green tissues is linked to photosynthetic rather than respiratory processes. The possibility therefore arises that esterification of phosphate in green tissues might in some way be associated with the presence or reduction of nitrate and hence the high level of inorganic phosphate in the ammonia grown plant be a consequence of a limiting of the esterification in the absence of nitrate.

It was argued that if the preponderence of phosphate esterification in the shoot is dependant upon the presence of both light and nitrate, then by feeding

nitrate to ammonia grown plants in light and in darkness, it might prove possible to show the formation of organic phosphate in the former case only.

MATERIALS AND METHODS:

Plants.

Seedlings of tomato var. Bonny Best were raised and transferred to water cultures in the manner described for earlier experiments.

Experimental Design and Harvesting.

Eighteen ammonium and eighteen complete cultures were set up with the composition of the solutions as described for the previous experiment. Culture solutions were maintained at pH 6.4 by daily additions of appropriate quantities of an N/50 solution of sodium hydroxide following titration of a sample of the culture solution at a glass electrode.

After twenty four days when the plants were showing five true leaves, half the nitrate and half the ammonia grown plants were placed in darkness by covering them with a wooden box painted black on the inside, the remainder being exposed to normal daylight. At the time of placing in the dark, half of the ammonia cultures to be placed in the dark and half of the ammonia cultures remaining in the light were fed nitrate, 5.0 mls. of a 10% solution of potassium nitrate per culture, giving a

final nitrate concentration of 0.002M.

At the time of applying treatments and at subsequent intervals of 14, 36, 48 and 59 hours from this time, four plants were taken at random from each treatment, and the shoots weighed and analysed for inorganic phosphate, soluble and insoluble organic phosphate content. The original intention was to carry out harvests at 12, 24, 36 and 48 hours, in order to cover the time period during which a change in the organic phosphate content was thought likely to occur, the time taken in dealing with the harvested samples prevented this.

Analyses.

Harvested shoots were ground in a pestle and mortar with cold 3% trichloracetic acid, the homogenate was made up to a known volume with cold 3% trichloracetic acid and then centrifuged at 300g for 10 minutes.

The sediment was twice resuspended and centrifuged in cold 3% trichloracetic acid to remove all soluble phosphate, and was then transferred to a Kjeldahl flask and subjected to a perchlorate digestion. Estimates of total insoluble phosphate in this digest were made by the method of Strickland et al, 1956.

A sample of the supernatant following the first centrifugation was used immediately for the estimation of inorganic phosphate as described in the previous experiment.

A further sample of the supernatant from the

first centrifugation was placed in a Kjeldahl flask, evaporated to a small volume, and then digested with perchlorate, total phosphorus in this digest was estimated by the method of Strickland et al, 1956. Subtraction of the value for inorganic phosphate from the value obtained following this digestion gives a measure of the soluble organic phosphate present in the extracts.

Results:

Table XXXIV and Figure 14 show the change in inorganic phosphate content with time.

The data in Tables XXXIV, XXXV and XXXVI is difficult to interpret, largely as a result of the high variability in the fresh weights of the shoots analysed. The size of the experiment permitted the analysis of only four shoots from each treatment at any one of the chosen time intervals; four replicates are insufficient to permit a useful analysis of regression; yet on the other hand the shoots were too variable in size to express the data on a per shoot basis.

It was decided to express the data in terms of fresh weight so that the variation is minimised sufficiently for statistical treatment.

Under certain conditions the fresh weights of the shoots have changed in the course of the experimental period; thus in Table XXXV the soluble organic phosphate content of nitrate grown shoots in the light, has de-

TABLE XXXIV. INORGANIC PHOSPHATE CONTENT OF SHOOTS EXPRESSED AS MICROGRAMS PER GRAM FRESH WEIGHT. EACH VALUE IS BASED ON THE MEAN OF FOUR SHOOTS, WEIGHED AND ANALYSED FOR INORGANIC PHOSPHATE CONTENT.

Time from Commencement of treatments. Hours.	LIGHT. Nitrate.	Ammonium.	Ammonium. fed Nitrate.	DARK. Nitrate.	Ammonium.	Ammonium. fed Nitrate.
0	263.4	334.2	334.2	263.4	334.2	334.2
14	263.0	334.7	328.2	296.1	361.9	333.7
36	267.0	370.8	335.9	361.7	419.3	370.5
48	275.2	410.7	366.8	416.0	466.0	393.9
59	293.9	416.0	369.9	465.5	481.7	464.2

TABLE XXXV. SOLUBLE ORGANIC PHOSPHATE CONTENT OF SHOOTS EXPRESSED AS MICROGRAMS PER GRAM FRESH WEIGHT. EACH VALUE IS OBTAINED AS THE MEAN TOTAL SOLUBLE PHOSPHATE CONTENT OF FOUR SHOOTS MINUS THE MEAN INORGANIC PHOSPHATE CONTENT OF THE SAME FOUR SHOOTS DIVIDED BY THEIR MEAN FRESH WEIGHT.

Time from Commencement of Treatment Hours.	LIGHT			DARK		
	Nitrate.	Ammonium.	Ammonium. fed Nitrate.	Nitrate.	Ammonium.	Ammonium. fed Nitrate.
0	169.2	101.5	101.5	169.2	101.5	101.5
14	155.1	121.8	110.1	165.3	100.0	104.8
36	126.5	119.2	119.5	149.9	121.3	111.3
48	141.3	113.0	125.4	144.2	133.0	123.7
59	143.6	10663	140.0	140.1	144.0	136.2

TABLE XXXVI. INSOLUBLE ORGANIC PHOSPHATE CONTENT OF SHOOTS EXPRESSED AS MICROGRAMS PER GRAM FRESH WEIGHT. EACH VALUE IS THE MEAN OF FOUR SHOOTS ANALYSED DIVIDED BY THE MEAN FRESH WEIGHT OF THE FOUR SHOOTS.

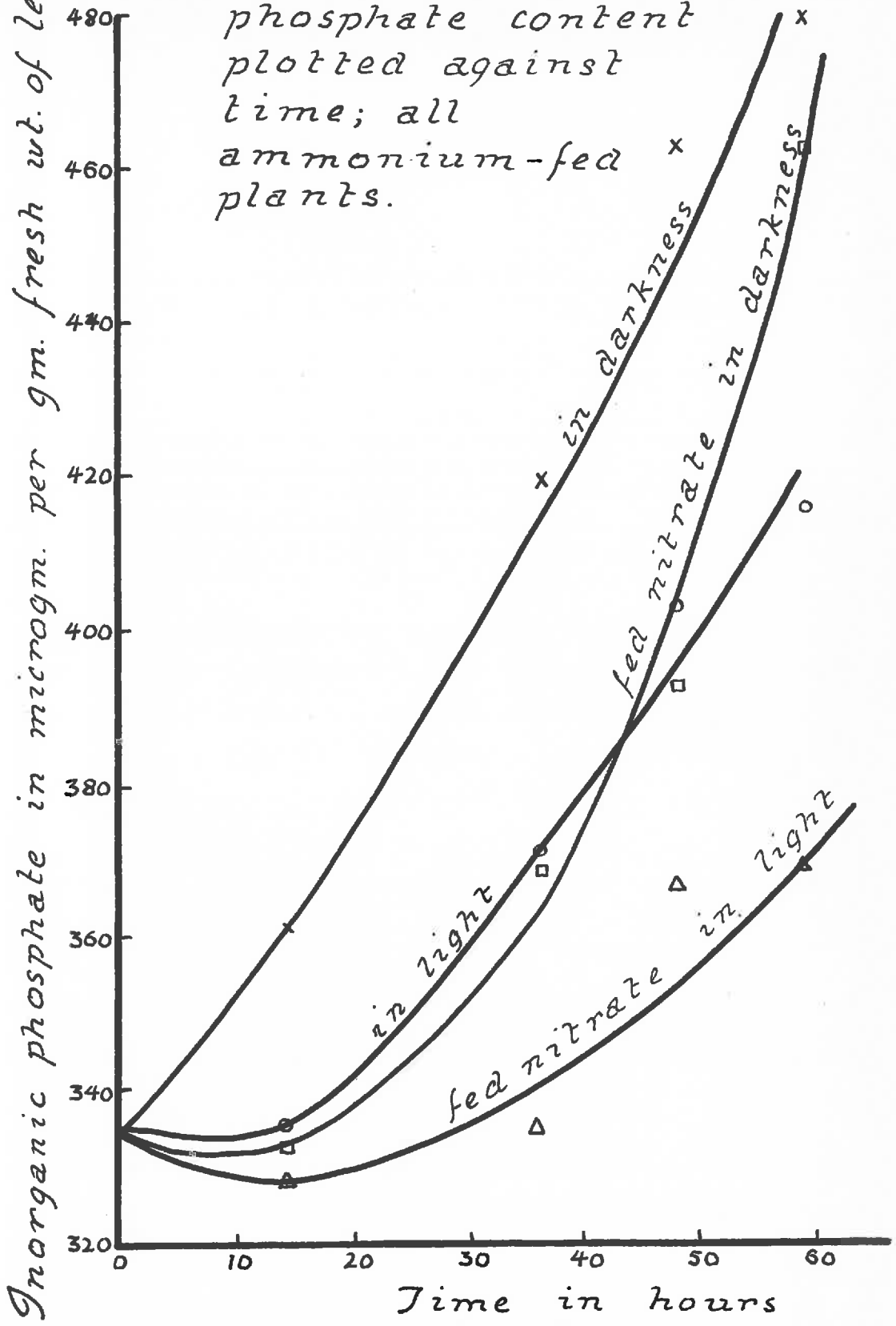
Time from Commencement of Treatment Hours.	LIGHT.			DARK.		
	Nitrate.	Ammonium.	Ammonium. fed Nitrate.	Nitrate.	Ammonium.	Ammonium. fed Nitrate.
0	232.4	221.6	221.6	232.4	221.6	221.6
14	231.4	215.5	240.0	199.7	200.5	240.3
36	226.8	210.6	250.5	134.6	180.0	225.6
48	199.3	245.9	257.4	91.3	143.1	165.2
59	188.1	269.5	294.2	78.8	132.3	99.0

creased slightly with time, and a similar change in the insoluble phosphate level, Table XXXVI, is seen to have occurred. In fact, however, the absolute amounts of soluble and insoluble organic phosphate in nitrate-receiving shoots grown in the light has increased during the fifty-nine hours of the experiment, though not at as fast a rate as the increase in fresh weight of the shoots. Similarly the small increased in the soluble organic phosphate content of ammonia-receiving shoots placed in the dark are believed to be the consequence of a slight decrease in the fresh weight of the shoots in the dark.

Plants placed in darkness:

An outstanding feature of this experiment, and one which, in part at least, defeats the original purpose of the experiment, is the profound effect of prolonged darkness on the level of the different phosphate fractions in the shoot. Thus in Table XXXV, in all three cases, i.e. nitrate present throughout, ammonia present throughout, and nitrate supplied to plants previously receiving only ammonia, absence of light resulted in a sharp, statistically significant, rise in the level of inorganic phosphate in the shoot tissues. As previously noted, plants placed in the dark did show a slight decrease in fresh weight during the experiment; this change however, is quite small so that the rise in inorganic phosphate content represents an increase in the absolute amount.

Figure 14 – Inorganic phosphate content plotted against time; all ammonium-fed plants.



As is seen in Table XXXV, the level of soluble organic phosphate in shoots placed in darkness remains approximately constant, the level of insoluble phosphate on the other hand falls rapidly. Apparently the above mentioned increased inorganic phosphate content is derived from the insoluble phosphate fraction. This result clearly warrants further investigation, it does however, constitute a distinct problem which is beyond the scope of the present work.

The trichloracetic insoluble phosphate probably consists mainly of phospholipids, nucleic acids, nucleo proteins and phosphoproteins; it would be of considerable interest to know whether one or all of these groups of compounds represents the source of inorganic phosphate released, and in this connection the question arises as to whether proteolysis is involved in the break-down of the insoluble fraction? A further problem is as to whether the inorganic phosphate released is the product of a direct hydrolysis of the insoluble phosphate fraction or whether the insoluble phosphate is first converted to the soluble organic phosphate fraction, consisting probably in the main of sugar esters and nucleotide esters, which are then subsequently catabolised with the release of inorganic phosphate? In this connection it is of interest to note that the lowering of the insoluble phosphate level is greatest in shoots receiving nitrate, although the

level of soluble organic phosphates is not appreciably altered in these plants; indeed in the instance of ammonia grown plants fed nitrate there is a suggestion, which does not, however, reach significance, of an increase in the soluble organic phosphate content of the plants. Now, as noted in the introductory discussion, the studies such as those of Clark and Shive, 1934, and of Yemm and Willis, 1958, indicates that nitrate supplied to roots evokes an increase in the rate of respiration, such a respiratory change might reasonably be expected to result eventually in an expending of the soluble phosphates. It would therefore seem possible that the insoluble phosphate fraction has been largely concerned with the maintaining of the soluble organic phosphate level; in this connection it would be of interest to know whether the increasing "level" of A.T.P. in cells of Chlorella cultured in the dark, and in which the rate of respiration was also increasing, Synett, 1958, was accompanied by changes in the insoluble phosphate content of the cells.

A further tentative argument might be based on these results: it was noted in the introduction that the work of Takahashi, 1956, on E. coli under anaerobic conditions, and of Nicholas, 1958, on nitrite reductase from N. cyanea, indicated a coupling of phosphorylation to nitrate reduction. It might be that the rate of nitrate reduction in green tissues being greater in the light,

Burstrom, 1943, then a coupled phosphorylation would also increase. It would be of interest to examine the rate of organic phosphate formation and of nitrate reduction at different light intensities; providing that corrections could be made for such factors as the possible effect of light intensity on the rate of respiration.

Plants growing in daylight.

In daylight, shoots grown in ammonium culture and subsequently fed nitrate showed an increased inorganic phosphate content, but this increase is not so great as in shoots remaining in ammonium culture, Table XXXIV. From Tables XXXV and XXXVI it is seen that there is an increase of organic phosphate in plants receiving nitrate, this increase which reaches significance after 48 hours, is present in both the trichloracetic soluble and insoluble fractions.

EXPERIMENT XIII. The Effect of Feeding Nitrate on Phosphate Esterification in Ammonia grown Plants.

INTRODUCTION:

Experiment XII indicated that nitrate fed to ammonia-grown plants in daylight, stimulates organic phosphate formation in the shoot. The purpose of the following experiment is to repeat the feeding of nitrate, examining different regions of the shoot in an attempt

to more specifically locate the esterification.

MATERIALS AND METHODS:

Plants.

Seeds of tomato var. Bonny Best were germinated and the seedlings transferred to water cultures as described for earlier experiments.

Culture Solutions.

Ammonium culture solutions were made up as described for Experiment IX

Experimental Design and Harvesting.

Twenty ammonium cultures were set up, the pH of the solutions was maintained at pH 6.5 by daily additions of appropriate quantities of an N/50 solution of sodium hydroxide, following titration of a sample of the culture solution at a glass electrode. Ammonium chloride was added to the cultures periodically in order to maintain the nitrogen supply at approximately 4×10^{-4}M. After twenty days 5.0 mls. of 10% potassium nitrate solution was added to eight of the cultures to bring the nitrate content to 0.0025M. At the time of application of the nitrate sixteen shoots were harvested at random from the remaining cultures, i.e. those not receiving nitrate. Because of the time taken for changes to occur in Experiment XII, intervals of twenty-four and forty-eight hours were selected for the harvests in the present experiment, at these times thirty-two plants were harvested, sixteen

from the nitrate-receiving group and sixteen from the group remaining on ammonia. During periods of darkness subsequent to the feeding of nitrate, the plants were illuminated at a light intensity of approximately 75 lumens per square foot, by three 300 watt lamps held above the plants; all plants were not equally illuminated by this method but an attempt to avoid differences due to gradients of light intensity was made by the random harvesting of shoots.

Analyses.

Leaf laminae only, from the harvested shoots were analysed; these were divided arbitrarily into two groups;

(a) the laminae of the first two true leaves;

(b) the laminae of all the younger leaves.

The two groups of leaves were weighed and analysed separately. Of the sixteen shoots harvested from each treatment at each time interval, eight were analysed for total phosphate content and eight for inorganic phosphate content. The organic phosphate content of the leaves was calculated as the difference between the total and the inorganic phosphate content. Inorganic and total phosphate were determined as described for Experiment XI, Chapter II.

RESULTS:

The mean fresh weights of the leaf laminae analysed are given in Table XXXVII.

TABLE XXXVII. FRESH WEIGHTS OF LEAF LAMINAE IN GRAMS. EACH VALUE IS THE MEAN WEIGHT OF EIGHT SAMPLES.

Time in Hours.	Ammonia grown plants.				Plants fed nitrate.			
	Leaves 1 & 2.		Young Leaves.		Leaves 1 & 2.		Young Leaves.	
	Leaves for Pi analysis.	Leaves for Po analysis.	Leaves for Pi analysis.	Leaves for Po analysis.	Leaves for Pi analysis.	Leaves for Po analysis.	Leaves for Pi analysis.	Leaves for Po analysis.
0	0.402	0.407	0.401	0.412	0.402	0.407	0.411	0.412
24	0.411	0.426	0.435	0.429	0.420	0.417	0.455	0.447
48	0.417	0.408	0.482	0.472	0.409	0.430	0.639	0.645

From Table XXXVII it is seen that the feeding of nitrate markedly enhanced the fresh weight of young leaves, but no great change in fresh weight of leaves one and two.

In leaves one and two of ammonia grown plants, there is a statistically significant increase in inorganic phosphate content, Table XXXIX, and inorganic phosphate "concentration", Table XLII, and a decrease in organic phosphate, during the forty-eight hours of the experiment. The total phosphate content of these leaves does not change significantly during this period, Tables XXXVIII and XLI, and changes in the proportions of inorganic and organic phosphate are indicated by the Pi : Po ratios, Table XXXIX, from 2.42 at zero time to 3.86 after forty-eight hours. The reason for this increase in inorganic phosphate, possibly related to the decrease in organic phosphate, in the older leaves of ammonia grown plants is not known: the corresponding leaves of plants receiving nitrate do not however, show a similar change. The total, inorganic and organic phosphate content of the first two true leaves of nitrate-receiving plants remains approximately constant throughout the experimental period, unless there are shorter-term fluctuations not detectable because of the twenty-four hour intervals between harvests. It may be that the presence of nitrate has inhibited a process or processes of hydrolysis in these leaves, by which inorganic phosphate is released; or again has induced a

TABLE XXXVIII. TOTAL PHOSPHATE CONTENT OF LEAVES IN MICROGRAMS OF P PER SAMPLE. EACH VALUE IS THE MEAN OF EIGHT SAMPLES ANALYSED.

Time in Hours.	Ammonia grown plants.		Plants fed nitrate.	
	Leaves one and two.	Young leaves.	Leaves one and two.	Young leaves.
0	312.2	324.3	312.2	324.3
24	327.9	346.8	295 .7	381.0
48	314.9	412.8	2948	602.7

TABLE XXXIX. INORGANIC AND ORGANIC PHOSPHATE CONTENT OF LEAVES ONE AND TWO IN MICROGRAMS PER SAMPLE. EACH INORGANIC PHOSPHATE IS THE MEAN OF EIGHT SAMPLES ANALYSED, THE ORGANIC PHOSPHATE VALUES ARE OBTAINED BY SUBTRACTING INORGANIC FROM TOTAL PHOSPHATE.

Time in Hours.	Ammonia grown plants. Inorganic phosphate (Pi)	Organic Phosphate (Po)	Ratio Pi:Po.	Plants fed Nitrate. Inorganic phosphate (Pi)	Organic phosphate (Po	Ratio Pi:Po
0	221.0	91.2	2.42	221.0	91.2	2.42
24	233.9	94.0	2.49	211.3	84.4	2.50
48	250.1	64.8	3.86	206.4	88.4	2.34

TABLE XL. INORGANIC AND ORGANIC PHOSPHATE CONTENT OF YOUNG LEAVES IN MICROGRAMS PER SAMPLE. EACH INORGANIC PHOSPHATE VALUE IS THE MEAN OF EIGHT SAMPLES ANALYSED. ORGANIC PHOSPHATE VALUES ARE OBTAINED BY EXTRACTING INORGANIC FROM TOTAL PHOSPHATE.

Time in Hours.	Ammonia grown plants. Inorganic phosphate(Pi)	Organic phosphate(Po)	Ratio Pi:Po	Plants fed nitrate. Inorganic phosphate(Pi)	Organic phosphate(Po)	Ratio Pi:Po.
0	225.6	98.7	2.29	225.6	98.7	2.29
24	246.8	100.0	2.47	219.8	161.2	1.36
48	283.0	129.8	2.18	236.6	366.1	0.65

TABLE XLI TOTAL PHOSPHATE CONTENT OF LEAVES EXPRESSED AS MICROGRAMS P PER GRAM FRESH WEIGHT.

Time in Hours.	Ammonia grown plants. Leaves one and two.	Ammonia grown plants. Young leaves.	Plants fed nitrate. Leaves one and two.	Plants fed nitrate. Young leaves.
0	767.1	787.2	767.1	787.2
24	769.8	807.8	709.0	852.3
48	771.8	874.5	771.8	934.4

TABLE XLII. INORGANIC AND ORGANIC PHOSPHATE CONTENT OF LEAVES ONE AND TWO EXPRESSED AS MICROGRAMS PER GRAM FRESH WEIGHT.

Time in Hours.	Ammonia grown plants. Inorganic phosphate(Pi)	Organic Phosphate(Po)	Ratio Pi:Po.	Plants fed nitrate. Inorganic phosphate(Pi)	Organic Phosphate(Po)	Ratio Pi:Po.
0	549.8	217.3	2.53	549.8	217.3	2.53
24	569.0	200.8	2.83	503.0	206.0	2.44
48	592.8	172.0	3.49	504.6	267.2	1.89

TABLE XLIII. INORGANIC AND ORGANIC PHOSPHATE CONTENT OF YOUNG LEAVES EXPRESSED AS MICROGRAMS PER GRAM FRESH WEIGHT.

Time in Hours.	Ammonia grown plants. Inorganic phosphate (Pi)	Organic Phosphate (Po)	Ratio. Pi:Po.	Plants fed nitrate. Inorganic. phosphate (Pi)	Organic. Phosphate (Po)	Ratio Pi:Po.
0	562.7	224.5	2.51	562.7	224.5	2.51
24	567.3	240.5	2.36	505.3	347.	1.46
48	587.1	287.4	2.04	370.3	564.1	0.66

process or processes of organic phosphate formation at a rate which balances the hydrolytic inorganic phosphate formation.

In the young leaves of ammonia grown plants there is a statistically significant increase in inorganic phosphate content of approximately 57 micrograms of phosphorus per sample, Table XL, during the experiment. This increase is proportional to the amount of growth, since the inorganic phosphate content per unit fresh weight of young leaves does not increase significantly in the course of the experiment; the ratio of inorganic to organic phosphate has fallen from 2.51 to 2.04 during the forty-eight hour period - this change is due to a small increase in organic phosphate "concentration", Table XLIII. The increase in total phosphorus content of the young leaves must have come from the stems, roots or represent newly taken up phosphorus if it is accepted that there is no suggestion from the data, of its being formed from phosphorous present in the older leaves.

The young leaves of plants receiving nitrate show a sharp increase in total phosphorous content of approximately 278 micrograms in forty-eight hours, Table XXXVIII. This increase is almost entirely in the organic phosphate fraction, Table XL, the Pi : Po ratio falling from 2.29 to 0.65 in forty-eight hours. After only twenty-four hours from the time of feeding nitrate, there has

occurred a statistically significant increase of 60 micrograms in the organic phosphate content of the young leaves, an increase which is not in proportion to the increase in fresh weight, Table XLI. From Table XLIII it is seen that in plants receiving nitrate, the inorganic phosphate content of the tissues is increasing much more slowly with increasing fresh weight than is the organic phosphate content. An interesting difference in comparison with Experiment XI, Chapter II, is that total phosphate content is increasing more rapidly in relation to fresh weight in plants receiving nitrate; in the earlier experiment whole shoots were analysed so that increases in the phosphate content of the young leaf laminae may not have been detected because of falls in the phosphate level in other parts of the shoot.

The question now arises as to whether this organic phosphate formation represents an esterification coupled to nitrate reduction and/or whether nitrate has effects on other reactions which directly or indirectly affect organic phosphate formation. It is of theoretical interest to make certain assumptions relating to a possible coupled phosphorylation in order to derive results for comparison with the data obtained.

Let us assume

(i) that in higher plant cells all assimilated nitrate is reduced along an "inorganic pathway" to ammonia;

(ii) that at two points along this sequence a molecule

of phosphate is esterified.

Then, in the absence of other changes evoked by nitrate there would be 2 moles of phosphate esterified per mole of nitrate reduced. By carrying out feeding experiments with varying concentrations of nitrate, making careful measurement of rates of nitrate consumption and organic phosphate turnover it might be possible to decide whether such a mechanism as the above mentioned could account for the amount of "extra" phosphate esterification which occurs following addition of nitrate.

The data obtained in this experiment is inadequate for the type of calculation mentioned above; certain general estimates may however, be made:-

Young leaves of plants receiving nitrate showed an increase in fresh weight of 0.233 gms. in forty-eight hours.

Young leaves of plants receiving ammonia only, showed an increase in fresh weight of 0.060 gms. in forty-eight hours.

Therefore the "extra" increase in fresh weight due to feeding nitrate = (0.233 - 0.060) gms. = 0.173 gms.

The nitrogen content of the young leaves is probably of the order 0.5% of the fresh weight = 0.000865 gms.

Young leaves of plants receiving nitrate showed an increase in organic phosphorus content of 0.000267 gms. in forty-eight hours.

Young leaves of plants receiving ammonia only,

showed an increase in organic phosphorus content of 0.000031 gms. in forty-eight hours.

Therefore the "extra" increase in organic phosphorus in leaves receiving nitrate = (0.000267 - 0.000031) = 0.000236 gms. of P.

If it is assumed that all the nitrogen contained in the new leaf tissues has come from nitrate reduced in the forty-eight hour period, then it may be said that the reduction of $\frac{865}{14} \times 10^{-6}$ gram atoms of nitrate nitrogen has resulted in the accumulation of $\frac{236}{31} \times 10^{-6}$ gram atoms of phosphorus in organic combination.
i.e. 62×10^{-6} moles of nitrate = 8×10^{-6} moles of phosphate esterified.

Thus if every organo phosphate group had "turned over" ten times, though there is no evidence to support such a value, in the forty-eight hour period, there would still be 6.2 moles of nitrate yielding 8.0 moles of organophosphate which is within the limits set in the first premises, i.e. such a mechanism could account for the amounts of organic phosphate formed. Many other objections may of course be raised, for example, it may be that phosphatase activity is higher in the tissues receiving only ammonia; measurements of phosphatase activity have not been made.

Possingham, 1954, found a high proportion of inorganic to organic phosphate in shoots of young tomato plants deficient in molybdenum; feeding of molybdenum to

deficient plants increased the organic phosphate content and dry weight of the shoots. Application of the above calculation to Possingham's data leads to an answer very close to the above result, i.e. 8 moles of nitrate reduced to one mole of phosphate esterified. Possingham interpreted his results in terms of an enhanced acid phosphatase activity under conditions of molybdenum deficiency, such as had been shown by Spencer, 1954.

Another way in which nitrate may be functioning is as an inducer or the source of an inducer for the adaptive formation of enzymes involved in phosphorylation processes. Candella et al, 1957, found a higher nitrate reductase activity in extracts from leaves of cauliflower when nitrate formed the inorganic nitrogen source than when ammonium salts were used; D.P.N.H. was used as an electron donor in the enzyme assays. Hewitt and Afridi, 1959, took leaf fragments of cauliflower, sunflower and white mustard, and infiltrated them with nitrate solutions under reduced pressure. The appearance of nitrite in the leaf fragments after a lag period of about an hour was taken to indicate the adaptive formation of nitrate reductase; the lag period may however, have been due to time required for passage of nitrate to the site of reduction. Again it may be that free nitrite only accumulates when the nitrite reducing system is saturated and that this does not happen until nitrate reduction has proceeded for an hour. As further evidence

for adaptive enzyme formation (the term "adaptive" is not defined, but from work on bacterial metabolism may be taken to mean the de novo synthesis of apo-enzyme from constituent amino acids), the inhibition of the production of nitrite, by such substances as actidione, polymyxin B-sulphate and 1 : 2 - dichloro-4-(p-nitrobenzenesulphonylamido)-5-nitrobenzene (D.C.N.S.) when infiltrated into leaf fragments along with the inducing nitrate, is cited. A similar absence of nitrite accumulation occurs when the above mentioned inhibitors are introduced with molybdenum to leaf fragments deficient in this component of the nitrate reducing system, this may be an indication that molybdenum deficiency limits apo-enzyme synthesis as well as being a component of the prosthetic group of the enzyme, it may on the other hand be due to the fact that the inhibitors used were not specific to protein synthesis in their action on living cells.

Rijvan, 1958, found nitrate reductase activity in extracts of excised embryos of Triticum vulgare only after twenty-hours pre-incubation of the embryos in a "sitting drop" culture containing 0.002M nitrate, sucrose and the essential mineral elements. Tang and Wu, 1957, germinated rice seedlings in M/15 Sorensen phosphate buffer in the dark at 30°C, if 0.002M nitrate was included in the buffer then after five days growth nitrate reductase activity (assayed as nitrite formed) was detectable in crude homogenates of

the seedlings. Extracts of seedlings raised in buffer solutions not containing nitrate showed no nitrate reductase activity; no investigation was made of the presence of cofactors of nitrate reduction in these extracts. From these separate pieces of work there is a suggestion of an adaptive formation of nitrate reductase, nitrate functioning as inducer, or source of inducer, but conclusive evidence is lacking.

As noted in the introductory discussion, Kinsky and McElroy, 1958, found an adaptive formation of T.P.N.H.-Cytochrome C reductase in Neurospora crassa, using nitrate as inducer. Marrè and Servettaz, 1958, by adding oxidised cytochrome C to illuminated suspensions of whole chloroplasts isolated from spinach and pea leaves showed the existence of a T.P.N.H.-Cytochrome C reductase activity in the chloroplasts. Cytochrome C probably does not occur in chloroplasts, James, 1958, but is probably replacing Cytochrome f, shown by Hill and Scarisbrick, 1958, to be present in chloroplasts, which has a similar redox potential.

The question then arises, that if T.P.N.H.-Cytochrome C (or f) reductase is a component of the electron transporting system of the chloroplast, to which system photosynthetic phosphorylation may be coupled, Arnon, 1958, and if this enzyme is formed in the presence of nitrate, then it might be possible to show a difference in the rate of photosynthetic phosphorylation and in the T.P.N.H.-Cytochrome C reductase activity, between chloroplasts isolated

from nitrate and ammonia grown plants. Clearly it is a big step from demonstrating any such differences, should they happen to exist, to arguments involving adaptive enzymes and impaded electron-transporting chains; the experiments to be reported represent a preliminary investigation.

EXPERIMENT XIV. THE ESTERIFICATION OF INORGANIC PHOSPHATE BY CHLOROPLASTS ISOLATED FROM NITRATE AND AMMONIA GROWN PLANTS.

INTRODUCTION:

It has been suggested that photosynthetic phosphorylation may account for a large proportion of the phosphate esterification which takes place in illuminated green leaves. This conclusion is based on the proportions of chloroplasts and mitochondrial material present in green leaves, and on the rates of photosynthetic phosphorylation by isolated chloroplasts and the rates of oxidative phosphorylation by isolated mitochondria. It is possible therefore that the high level of inorganic phosphate in the leaves of ammonia grown plants might be due to a less active phosphorylating system in the chloroplasts. It will be recalled that in Experiment XIII it was shown that the total phosphate content of the leaves of nitrate and ammonia grown plants was the same so that the inorganic phosphate present in ammonia grown plants could not have been due to an enhanced

uptake. A comparison was therefore carried out of photosynthetic phosphorylation by suspensions of whole chloroplasts, isolated from the leaves of nitrate and ammonia grown plants.

MATERIALS AND METHODS:

Plants.

Seed of tomato variety Sonny Boat was germinated and the seedlings transferred to water cultures as described for earlier experiments. Complete and ammonia cultures were set up, the composition of the solutions was as described for Experiment IX . After twenty-one days, fully expanded leaves from complete culture and ammonia grown plants were harvested and the midribs removed: the first four true leaves from nitrate grown plants were harvested, the first leaf having been fully expanded for about ten days, whilst the fourth leaf had only just become fully expanded. The first three true leaves from ammonia grown plants were fully expanded at the time of harvest, as with the leaves of nitrate grown plants they varied in time they had been expanded, over a period of about ten days.

Preparation of Chloroplasts.

Ten-gram samples of freshly harvested leaves from each treatment were ground in a mortar with 30 mls. of an ice-cold solution containing 0.2M sucrose, 0.02M potassium ascorbate, 0.1M Tris., and 0.002M E.D.T.A. at pH 7.5. The homogenate was strained through a double layer of cheese

cloth, and the resulting green suspension centrifuged at 100 xg for 5 minutes to remove unbroken cells and other debris. All centrifuging was carried out in an "International" refrigerated centrifuge at 0° C. The supernatant from this centrifuging was decanted, re-centrifuged at 1000 xg for 10 minutes and the resulting supernatant discarded. The remaining green pellet was resuspended in 30 ml. of a washing solution containing 0.23M sucrose, 0.02M potassium ascorbate, 0.002M E.D.T.A., and 0.04M Tris at pH 7.5. This suspension was centrifuged at 1000 xg for ten minutes and the supernatant discarded. This washing procedure was repeated a second time, after which the pellet was finally suspended in 10 mls. of a solution containing 0.18M sucrose, 0.05M potassium ascorbate, 0.002M E.D.T.A. and 0.04M Tris at pH 7.5.

The reaction to measure photosynthetic phosphorylation was carried out in a Brown-Warburg manometer vessel continuously shaken in a refrigerated water bath at 12° C. Some samples were illuminated by a 750 watt lamp held 50 cms. above the bath, a mirror was placed on the bottom of the bath in order to give a more even illumination. The reaction mixture contained 25 μ M. of KH_2PO_4, 300 μ M sucrose, 50 μ M glucose, 200 μ M Tris., 10 μ M $MgCl_2$, 3 μ M A.D.P., 0.3 μ M F.M.N. and 225 Kunitz units of hexokinase (Sigma crude preparation) contained in 0.5 mls. of water. The reaction was started by switching on the light and adding 0.5 ml. of

chloroplast suspension bringing the final volume to 3.0 mls., at pH 7.8.

The reaction was stopped (after 30 minutes in some samples and 60 minutes in others) by addition of 0.5 mls. of a 30% solution of perchloric acid.

Analyses.

Two methods were tried for estimating the amount of inorganic phosphate esterified.

(i) The reaction mixture was centrifuged, the supernatant decanted, and the residual inorganic phosphate estimated by the method of Strickland et al, 1956, as described for earlier experiments.

(ii) The second method used was based on the enzymic method of Slater, 1953, for the estimation of the glucose-6-phosphate formed by the reaction of glucose with the A.T.P. generated by the photosynthetic phosphorylation, catalysed by the hexokinase present in the reaction mixture. The method consists essentially in the conversion of the glucose-6-phosphate, by a mixture of the appropriate glycolytic enzymes, to glyceraldehyde-3-phosphate which is in turn enzymically converted to glycerol phosphate, according to the equation:-

Glyceraldehyde-3-phosphate + DPNH + Enzyme $\rightleftharpoons$ Glycerolphosphate + DPN^{-} + Enzyme.

The reaction is measured by the amount of D.P.N.H.

oxidised as indicated by the change in optical density at a wavelength of 340 m μ. This procedure met with difficulties due to the presence of a substance(s), probably leached from the chloroplasts, which brought about a non-enzymic oxidation of D.P.N.H. The interfering substance(s) were stable at temperatures of 80° C for 10 minutes they have not been further identified.

Chloroplast protein was estimated by the Ma and Zuazaga, 1942, modification of the Kjeldahl method.

RESULTS:

The data given in Table XLIV is based on the measurement of residual inorganic phosphate.

The variability between analyses was too high, and the change in amount of inorganic phosphate too small in relation to the total amount present, to make these measurements reliable. Table XLIV shows that, under the conditions described, there is a significant difference, P = 0.01, in the rates at which chloroplasts isolated from ammonia grown plants and from plants receiving nitrate carry out photosynthetic phosphorylation.

DISCUSSION:

If the observations contained in Table XLIV are the result of changes in the functioning of the chloroplasts in ammonia grown plants one might ask how much change has occurred.

TABLE XLIV. PHOTOSYNTHETIC PHOSPHORYLATION BY ISOLATED CHLOROPLASTS FROM THE LEAVES OF COMPLETE CULTURE AND AMMONIA GROWN PLANTS. EACH VALUE IS THE MEAN OF SIX SAMPLES INCUBATED. EXPRESSED AS MICROGRAMS OF PHOSPHORUS ESTERIFIED PER MILLIGRAM OF CHLOROPLAST PROTEIN.

Incubation time in minutes.	Complete Culture.		Ammonium Culture.	
	Light.	Dark.	Light.	Dark.
30	106.6	17.	23.	19
60	155.	16.	42.	20.

The variability between analyses was too high, and the change in amount of inorganic phosphate too small in relation to the total amount present, to make these measurements reliable.

In Experiment XI it was shown that changes in the levels of inorganic and organic phosphate occurred in the shoots of ammonia grown plants after seventeen days. Thus it is probable that after twenty one days, the time at which leaves were harvested in the present experiment, many metabolic reactions of the ammonia grown plants, including those of the chloroplasts, have become altered, though not as primary consequences of the ammonia treatment.

Considerations of this kind raise the question of the extent to which chloroplasts isolated from the two types of plant are physiologically comparable. Microscopic examination of the chloroplast suspensions before and after incubation indicated that the particles studied were of equal size and remained intact during incubation. Jagendorf, 1955, fractionated chloroplasts of spinach and tobacco by a density method; preliminary experiments revealed differences in enzyme activity between chloroplasts of different densities and it was also found that the proportion of chloroplasts in a particular density fraction varied according to the age of the leaves from which they were extracted.

Evidence of this type emphasises the possibility that the differences shown in Table XLIV may, in part at least, be due to differences in the proportion of chloroplasts of a particular physiological age and condition.

in nitrate and ammonia grown plants.

Different workers have observed widely differing rates of photosynthetic phosphorylation in suspensions of isolated chloroplasts, e.g. Arnon et al, 1957, and Avron et al, 1958. Such differences may in part, be due to the above mentioned condition of the chloroplasts; many other factors have however, been found to influence the rate of photosynthetic phosphorylation by isolated chloroplasts. These include, pH, osmotic pressure, the presence of oxygen and the presence of "co-factors" such as vitamin K, F.A.D., and ascorbic acid. The problem of "co-factors" may be particularly important in a comparative study; thus Arnon et al, 1958, indicated the possibility that several different phosphorylating systems may be functioning in isolated chloroplasts, e.g. :-

(1) A phosphorylation coupled to the reduction of T.P.N. which may be represented by the scheme:-

$$\text{Light} + \text{Chloroplasts} + \text{T.P.N.}^{+} + 2H_2O \longrightarrow \text{T.P.N.H.} + 2OH^{-}$$

$$\text{A.D.P.} + \text{Pi} \longrightarrow \text{A.T.P.}$$

(2) Phosphorylation(s) coupled to the oxidation of T.P.N.H.

$$\text{T.P.N.H.} \longrightarrow \text{T.P.N.}$$

$$x\ \text{A.D.P.} + x\ \text{Pi} \longrightarrow x\ \text{A.T.P.}$$

This latter system is particularly activated

by vitamin K, ascorbic acid and F.A.D., their addition stimulating photosynthetic phosphorylation but stopping the evolution of oxygen; this represents the so-called cyclic phosphorylation of Arnon et al, 1958. It may be that this cyclic phosphorylation represents an artificially constructed system; if this were so, then it may be a difference in this artificial system which accounts for the higher phosphorylating activity of chloroplasts from nitrate than from ammonia grown plants, rather than a difference in slower phosphorylation reactions which would never-the-less be the important ones in vivo. One method of approaching this problem would be to examine the relationship between the abundance of chloroplasts in a piece of tissue and the rate of inorganic phosphate esterification during a short period of illumination. Data obtained in this way could then be compared with rates of phosphorylation measured in isolated chloroplasts, when expressed on a per-chloroplast basis. The difficulty with the in vivo measurement and possibly with the in vitro measurement also, as discussed earlier, is that even if short time intervals are used, there may be other systems than photosynthesis affected by the illumination. treatment. Thus, for example, although the subject is a controversial one, Rabinowitch, 1945; data such as that of Decker, 1955, suggests that there are rapid changes in the rate of respiration following the illumination of green

leaves. It may be possible to specifically inhibit the respiratory system otherwise it may have considerable effects on the phosphate metabolism of the leaves during the period of investigation.

It may be that the observed difference between chloroplasts isolated from nitrate and ammonia grown plants is due to a modification of some part of the phosphorylating apparatus of the chloroplast which is actually of significance in vivo, and that it is this which accounts for the high level of inorganic phosphate in ammonia grown plants which was observed in earlier experiments.

One possibility is that there may be a modification of the electron transporting chain concerned in the oxidation of T.P.N.H. to which the esterification of phosphate is coupled. Kinskey and McElroy, 1958, showed T.P.N.H.-Cytochrome C reductase to be adaptively formed in Neurospora crassa using nitrate as inducer. Marrè and Servettaz, 1958, found T.P.N.H.-Cytochrome C reductase activity in chloroplasts isolated from the leaves of garden pea and spinach, and showed that there was probably a phosphorylation coupled to this reaction. It is possible therefore that if nitrate were the inducer for the adaptive formation of such a system in the chloroplasts of the tomato plant, then it might be the absence of this system which accounts for the restricted phosphorylating activity in chloroplasts isolated from plants grown with

only ammonium nitrogen.

With this possibility of an adaptive effect in view, an attempt was made to determine the T.P.N.H.-Cytochrome reductase activity in chloroplasts isolated from nitrate and ammonia grown plants, following the proceedure described by Yarrè and Servettaz, 1956. The presence of a powerful, and apparently non-enzymic cytochrome C - reducing activity in all chloroplast suspensions so far prepared has prevented measurement of the enzyme activity. This reducing activity was not readily leached from the chloroplasts by successive washing and centrifuging in buffer solutions, and is probably bound to the chloroplast structure. Whether this system has any significance in vivo is not known, it may be that the Cytochrome C is being brought into contact with reducing sites at which it would not normally occur in vivo.

EXPERIMENT XV. THE ASSIMILATION OF NITRATE LABELLED WITH N^{15}, BY ISOLATED WHOLE CHLOROPLASTS.

INTRODUCTION:

There would appear to be no reports in the literature, of nitrate reduction by isolated whole chloroplasts. It may be that nitrate reduction does not occur in chloroplasts or that the nitrate reducing enzymes are leached from the chloroplasts in the course of the proceedures

normally adopted for their extraction. Again it may be that the two reduction products by which nitrate reductase is normally assayed, i.e. nitrite and ammonia either do not occur (i.e. some other pathway of reduction may be involved), or may be themselves so rapidly utilised within the chloroplast that they are not readily detected in the free state. Blasakian and Chamova, 1949, detected glutamic dehydrogenase activity in isolated chloroplasts, although this activity was not so high as in the soluble protein fraction it may be sufficient to remove any free ammonia formed in the course of nitrate reduction. It is the purpose of the present experiment to demonstrate the reduction of nitrate in isolated chloroplasts by showing the passage of the nitrogen atom, labelled as N^{15}, from nitrate into the protein of the chloroplast.

MATERIALS AND METHODS:

Plants.

Seeds of tomato variety Sonny Best were germinated and the plants grown in water cultures as described for previous experiments.

Culture Solutions.

Six nitrate and six ammonium cultures were set up, composition of the culture solutions and control of solution pH were as described for Experiment IX

Assay of Nitrate Reduction.

Twenty one days after the setting up of the cul-

tures 20 grams of leaf laminae from nitrate and ammonia grown plants were separately harvested, and each sample ground in a pestle and mortar with 20 mls. of a cold solution containing 0.4% glucose, 0.02M K_2HPO_4, 0.02M $MgCl_2$, and 0.01% ascorbic acid, at pH 7.0. The harvested leaves from both nitrate and ammonia grown plants were in varying stages of expansion. The leaf grindates were squeezed through a double layer of cheese cloth and the filtrate centrifuged at 100 xg for 5 minutes. The resulting supernatants were then centrifuged at 2000 xg for 10 minutes. All centrifugings were carried out in an "International" refrigerated centrifuge at 0° C. The sediments from this centrifugation, which contained a large proportion of unbroken chloroplasts, were resuspended in 10 ml. aliquots of the above mentioned glucose buffer solution at pH 7.0 and maintained at 0° C., and re-centrifuged at 2000 xg for 10 minutes. The chloroplast pellets were finally resuspended in 20 ml. aliquots of the glucose-buffer solution at pH 7.0 and maintained at 0° C.

Samples of the chloroplast suspensions were incubated in stoppered boiling tubes of one inch diameter and six inches in length. One ml. aliquots of the chloroplast suspension were incubated with 27 μ moles of $KN^{15}O_3$ containing more than 99 atom percent N^{15}, in a final volume of 2.0 ml. at pH 7.0. During incubation the tubes were continuously shaken whilst held at an angle of forty-five degrees in a water bath maintained at 23° C. All

tubes were illuminated at a light intensity of approximately 100 lumens per square foot, by a 300 watt lamp suspended above the water bath.

Three samples of chloroplasts from nitrate grown plants and three samples from ammonia grown plants were incubated for 30 minutes and similar numbers of samples for periods of 60 and 120 minutes: control tubes were incubated at each time interval using chloroplast samples, boiled prior to the addition of $KN^{15}O_3$.

The reaction was stopped by addition of 1.0 mls. of 10% trichloracetic acid. The chloroplast proteins were then separated by centrifuging at 5000 xg for 15 minutes, and washed successively with 10 mls. cold 4% trichloracetic acid, 10 mls. 4% trichloracetic acid at 80° C. for 20 minutes and 10 mls. 0.2% hydrochloric acid at 70° C for 15 minutes. Following each washing the sample was centrifuged at 5000 xg for 15 minutes. The proteins were then oxidised with 1.0 ml. of performic acid prepared according to the method of Schram et al, 1954, some of the protein dissolved under this treatment and was reprecipitated by addition of 10 mls. of cold 5% trichloracetic acid. This procedure probably results in some loss of protein but is included in order to oxidise free - SH groups on the protein which constitute possible sites of a non-specific binding of N^{15}. The proteins were finally washed with 10 mls. of acetone followed by 10 mls. of 70% ethyl alcohol

at 60° C for 10 minutes.

The washed protein samples were transferred to microkjeldahl flasks and digested for 2.5 hours with 2.0 mls. of concentrated sulphuric acid and 0.67 grams of a catalyst consisting of 47 grams of potassium sulphate to one gram of copper sulphate. The digests were then transferred to a Pregle steam distillation apparatus, the ammonia liberated by addition of 10 ml. of 10% W/V solution of sodium hydroxide and steam distilled into 5.0 mls. 0.7N sulphuric acid. This procedure yielded approximately 0.6 mgms. of protein nitrogen per sample, as determined with the Nessler reagent, by the method of King, 1951. Each sample was made up to 1.0 mgms. of nitrogen by addition of 1.0 mls. of 0.0143M ammonium sulphate solution and concentrated to a volume of approximately 3 mls. by evaporation under an infra red lamp. Samples were then transferred to one limb of a Rittenberg tube; 2.0 mls. of a solution of sodium hypobromite prepared according to the Sims and Cooking, 1958, modification of the method of Rittenberg et al, 1939, were placed in the second arm of the Rittenberg tube. The tubes were then warmed to 50° C in a water bath and partially evacuated with a water pump, further evacuation to a pressure of 5×10^{-6} mms. of mercury was obtained by placing the tubes in a bath containing solid carbondioxide in alcohol and evacuating with a mercury diffusion pump.

The hypobromite solution was introduced into the sample by tipping, and the nitrogen liberated introduced into a mass spectrometer as described by Rittenberg et al, 1939. The abundance ratio (R) of the molecules $N^{14}N^{14}$: $N^{14}N^{15}$ was measured and converted into atom per cent of N^{15} by the formula:-

$$P = \frac{100}{2R+1}$$ where P is the atom per cent of N^{15}.

From this value the natural abundance of N^{15} is subtracted, and the data presented as atom per cent excess of N^{15}.

RESULTS:

As seen from Table XLV there is no incorporation of N^{15} into the protein of chloroplasts isolated from ammonia grown plants. There is a suggestion of N^{15} incorporation into the protein of chloroplasts isolated from nitrate grown plants, although the figures are erratic and do not show a progressive increase with time. The amount of N^{15} incorporated represents only about 0.12% of the N^{15} present in the incubation mixture or 0.1% of the total nitrogen present in the protein of a chloroplast sample.

Several factors may be responsible for the result obtained, e.g. it may be that the nitrate assimilating activity of the chloroplasts is normally low, that the activity of the system has been lowered by extraction by re-

TABLE XLV. ATOM PERCENT EXCESS OF N^{15} IN THE PROTEIN OF ISOLATED CHLOROPLASTS INCUBATED WITH $N^{15}O_3$. DATA FROM EACH SAMPLE IS QUOTED SEPARATELY.

Time of Incubation in Minutes.	Chloroplasts from Nitrate grown plants.	Chloroplasts from Ammonia grown plants
30	0.12	0.01
30	0.10	0.00
30	0.00	0.00
60	0.16	0.00
60	0.00	0.02
60	0.09	0.00
120	0.00	0.00
120	0.18	0.00
120	0.21	0.00

No incorporation of N^{15} was obtained with samples boiled prior to incubation.

moval of enzymes or co-factors, or that inhibitors have been introduced during extraction. Hisaakian et al, 1955a, 1955b, 1957, obtained incorporation of C^{14} glycine into the protein fraction by whole chloroplasts isolated from the leaves of Phaseolus. Washing of the chloroplasts with a sucrose phosphate buffer solution enhanced the rate of glycine incorporation an effect which the authors attributed to removal of an inhibitor in the course of washing. Stevenson et al, 1956, obtained incorporation of C^{14} D.L. leucine into protein by chloroplasts isolated from tobacco leaves: under aerobic conditions illumination of the chloroplast suspension during incubation doubled the rate of C^{14} incorporation into the protein fraction. In none of the above mentioned studies was a net synthesis of protein demonstrable. This may have been due to the small amounts of protein formed, or to the fact that protein synthesis was not occurring, but rather the single labelled amino acids were replacing non-labelled counterparts in otherwise intact protein chains. Reactions by which this incorporation of single amino acids may occur have been described by Wieland et al, 1955, and by Brenner et al, 1955. A similar situation may have prevailed in the present experiment, incorporation of an N^{15} labelled amino acid(s) into the chloroplast protein may have occurred without de novo synthesis of protein taking place. On the other hand it would seem likely that the treatment to which the chloro-

plast proteins were subjected prior to Kjeldahl digestion, would have removed any "bound nitrate". This being so it may be said that nitrate reduction and presumably incorporation of the reduction products into amino acids has probably taken place in the isolated chloroplasts during the period of incubation, as to whether de novo synthesis of protein has also occurred, no conclusion can safely be drawn.

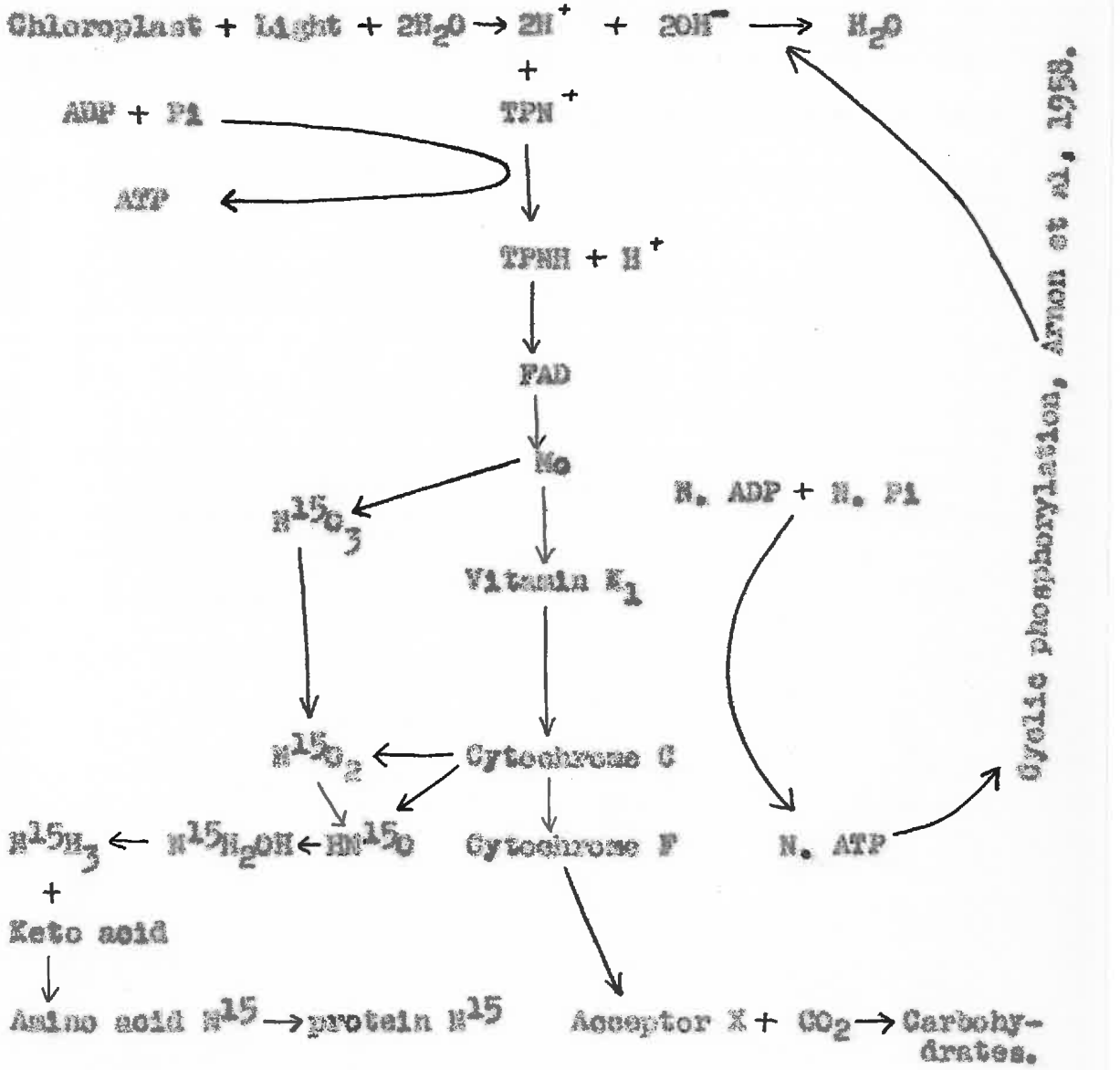
DISCUSSION:

In Experiment XV evidence was obtained of a lower rate of photosynthetic phosphorylation in chloroplasts isolated from ammonia grown plants than in chloroplasts isolated from nitrate grown plants. In Experiment XVI tentative evidence is obtained of the reduction of nitrate by chloroplasts isolated from nitrate grown plants but not by chloroplasts from ammonia grown plants, in this experiment there is no information on whether or not the nitrate reduction is light dependent. In the phosphorylation experiment it is presumed that no nitrate was present unless it was a small amount contained within the chloroplasts isolated from nitrate grown plants, thus it is unlikely that the observed phosphorylation is coupled to an electron transporting system in which nitrate is functioning as a terminal electron acceptor.

The diagram on page 197 is a hypothetical scheme attempting to relate an inorganic pathway of nitrate reduc-

tion to the electron transporting system of the chloroplast, concerned in the oxidation of T.P.N.H. The coupling of A.T.P. formation to the reduction of T.P.N. follows the evidence of San Pietro and Lang, 1958. The F.A.D. stimulated oxidation of T.P.N.H. concurrent with the reduction of Cytochrome C is based on the evidence of Marrè and Bervettaz, 1958. The inclusion of vitamin K_1 in this electron transporting chain and the evidence for coupled phosphorylation reactions is based on the studies of Arnon, Whatley and Allen, 1955, and 1957. The introduction of Molybdenum between F.A.D. and vitamin K might explain why the feeding of molybdenum to deficient shoots of tomato resulted in a rapid esterification of phosphate and a rapid increase in the rate of photosynthesis, Possingham, 1954. Whatley et al, 1951, found molybdenum to be present in extracted chloroplasts although it is uncertain whether the metal is normally concentrated in this portion of the cell. The absence of a phosphorylation coupled to the reduction of nitrate by soluble extracts, may be explained on this scheme, if it is supposed that the formation of this soluble system, is the consequence of a breaking of the intact electron transporting system at the point of coupling to vitamin K, during the process of extraction.

The reduction of nitrite and hyponitrite coupled to the cytochrome system is largely hypothetical, based on analogy with the situation in Neurospora. However, if it be that the reduction of $N^{15}O_3$ observed in the present


Chloroplast + Light + $2H_2O \rightarrow 2H^+ + 2OH^- \rightarrow H_2O$
+
ADP + Pi
TPN^+
ATP
TPNH + H^+
FAD
Mo
$N^{15}O_3$
Vitamin K_1
N. ADP + N. Pi
$N^{15}O_2$
Cytochrome C
$N^{15}H_3 \leftarrow N^{15}H_2OH \leftarrow HN^{15}O$
Cytochrome F
N. ATP
Cyclic phosphorylation, Arnon et al. 1958.
+
Keto acid
Amino acid $N^{15} \rightarrow$ protein N^{15}
Acceptor X + $CO_2 \rightarrow$ Carbohydrates.

experiment passed over such an inorganic pathway then it might be expected that the process of reduction would be inhibited by an uncoupling reagent such as dinitrophenol. The reduction of cytochrome f by Cytochrome C is also not demonstrated in chloroplasts, but is thermodynamically possible, the respective redox potentials being 0.37v. and 0.26v., Hill and Scarisbrick, 1956.

The absence of $N^{15}O_3$ reduction and lowered rate of photosynthetic phosphorylation in chloroplasts isolated from ammonia grown plants may be explained by supposing that nitrate is the natural inducer for an apo enzyme protein concerned between the oxidation of T.P.N.H. and the reduction of Cytochrome C. This protein may be identical with or distinct from the nitrate reducing system also adaptively formed in response to the presence of nitrate. Experiments are planned to examine the nitrate reducing activity of chloroplasts isolated in non-aqueous media in which it is hoped that the leaching of soluble enzymes will be lessened. Should this prove possible, it is intended to fractionate the chloroplast proteins with a view to following the incorporation of inorganic nitrogen compounds into particular fractions.

G E N E R A L D I S C U S S I O N.

In the introduction evidence concerning nitrate assimilation in the major plant groups was examined, with the notable exception of the Bryophyta, Pteridophyta and Gymnosperms on which relatively little work has been done.

Information from studies on widely differing types of plant can be used in the formulating of hypotheses concerning the metabolism of any particular species. In the formation and experimental testing of a hypothesis however attention must be given to the fact that information concerning any particular group of organisms may be considerably biased because of the particular type of experiment to which the species are amenable.

Arising out of preliminary work on ascorbic acid in relation to nitrate assimilation, a difference in growth between young tomato plants receiving nitrate or ammonium ions as the source of nitrogen was observed. Subsequent work is concerned with the reasons for this difference.

The data in Experiment IX indicates that toxic effects of ammonia are unlikely to be responsible

for the small amount of growth of ammonia grown plants. Much could be done to improve this experiment but it would seem a reasonable conclusion that when ammonia is supplied at a concentration never in excess of 4×10^{-4} N, the lessened growth is not due to "toxic" effects of ammonia, either by attack of the ammonium ion on vulnerable sites within the cell or, as originally suggested by Prianishnikov, by the using-up of the carbohydrate reserves of the plant in amide formation.

An important aspect of this problem is that growth on the two forms of inorganic nitrogen might be the same if optimal environmental conditions were provided in each instance. Several workers have reported different pH optima for plants grown in nitrate and ammonium cultures, Experiment VIII, however in which plants were grown in culture solutions at various pHs, failed to show any pH at which the growth of plants in ammonium cultures was as great as that of plants in nitrate cultures.

The influence of light on nitrate assimilation has been the subject of many investigations, e.g. Burstrom 1942 and Stoy 1955 having provided evidence

for a relationship between light intensity and light quality, and nitrate assimilation in green tissues. This problem has not been examined in the present work but it is perhaps worth noting that seasonal fluctuations in light intensity between different experiments resulted in considerable differences in growth. These differences were greater in nitrate than in ammonia grown plants e.g. Tables XXIII and Table XXVI. The data from algal studies, in so far as it might be applicable to higher plants, indicates the complexity of the interaction between nitrate and carbon dioxide assimilation. There is however no effect comparable to the present observation on tomatoe plants, from algal work i.e. growth on ammonium nitrogen is as great as or greater than growth on nitrate under the particular conditions most generally used. Tables XXIX and XXX show that the divergence in fresh and dry weights between nitrate and ammonia grown plants occurs in the shoots before the roots. Whilst this may be the consequence of a difference in "activity" between the roots of nitrate and ammonia grown plants before there is any apparent difference in morphology or dry weight, the difference may on the other hand be due to a direct effect on the metabolism of the shoot. In

relation to this problem of the location of primary changes leading to slower growth, it is possible that ammonia translocation to the shoot is a limiting factor in growth. However the ready formation of amides in the roots of plants receiving ammonia, and tentative evidence for these compounds as normal forms in which nitrogen is translocated, Oland and Yen 1956, suggests that translocation is probably not limiting in ammonia grown plants.

The enhanced growth of the shoot, particularly when light intensity is not limiting suggests the possibility of a difference in photosynthesis between nitrate and ammonia grown plants. This possibility is further supported by the finding, Experiment X, of a higher starch and lignin content in nitrate than in ammonia grown plants. Doman and Vaklinova, 1958 have similarly found a lower starch content in ammonia than in nitrate grown plants of spinach.

Gericke 1943 reported a lower phosphate requirement for plants grown on ammonia than for plants grown on nitrate. Arnon 1939 found a higher phosphate content in barley plants grown on ammonium cultures than in plants grown in nitrate cultures. The relative

proportions of chloroplastic to mitochondrial material in green leaves and the rates of photosynthetic phosphorylation in isolated chloroplasts as compared to the rates of oxidative phosphorylation in isolated mitochondria have led to the suggestion that a large proportion of the phosphate esterification occurring in illuminated green leaves occurs by photosynthetic phosphorylation. In Experiment XI the total phosphate content of tomato plants receiving nitrate and ammonium nitrogen is measured and at the same time the distribution of phosphate between the organic and inorganic fractions in nitrate and ammonia grown plants is examined. No difference was found between the total phosphate content of nitrate and ammonia grown plants but a high inorganic phosphate content and high ratio of inorganic to organic phosphate was found in ammonia as compared to nitrate grown plants. The difference in proportions of inorganic and organic phosphate between nitrate and ammonia grown plants occurred in the shoots two to three days before there were any changes in the roots, again suggesting the possibility of an effect on photosynthesis in the shoot before the metabolism of the root is affected. Nitrate fed to ammonia grown plants resulted in a considerable esterification of phosphate within twenty-four hours, esterification occurred in young leaves before mature, i.e. fully expanded, leaves, Experiment XIII.

Attempts to decide whether this phosphorylation was light-dependent, Experiment XII proved unsuccessful, however it is probable that use of an isotope technique in order to shorten the duration of the experiment, might make this possible. Calvin et al 1959 have reported that Chlorella suspensions supplied with nitrate produced a large quantity of sugar-phosphates during a ten-minute period of photosyathes, suspensions supplied with ammonium ions on the other hand formed only small quantities of sugar-phosphates but higher levels of amino acids. The total phosphate esterified in the two cases was not given.

The finding, Experiment XIV of a higher rate of photosynthetic phosphorylation in chloroplasts isolated from nitrate grown plants than in chloroplasts isolated from ammonia grown plants, provides further evidence that this phosphorylating system may be altered in ammonia grown plants. Much more experimental work would however be necessary in order to show that this system was the first to become altered in the ammonia grown plant.

As to how this effect on photosynthetic phosphorylation might occur there are many possibilities. The

fact that this difference in rate of phosphorylation between chloroplasts isolated from nitrate and from ammonia grown plants occurs in both cases in the absence of nitrate, makes it unlikely that phosphorylation is a reaction coupled directly to nitrate reduction. A further interesting possibility is that nitrate is functioning as an inducer for some adaptively formed component involved in the photosynthetic phosphorylation process. In the introduction and in relation to Experiment XIV the evidence for nitrate induction of adaptive enzymes in higher plants was examined and found to be suggestive of such an effect but inconclusive. From micro-organisms and fungi however there is good evidence of nitrate functioning as an inducer of adaptive enzyme formation. From N. crassa a nitrate reductase and a TPNH - cytochrome reductase, both induced by nitrate and possibly consisting of a common apo-enzyme, have been isolated. Marrè and Servattaz 1958 found TPNH - Cytochrome c reductase in isolated chloroplasts of Pisum sativum, to this system a light stimulated phosphorylation reaction was shown to be coupled. The possibility therefore arises of such a system in the chloroplast being adaptively formed in the presence of nitrate as an inducer, or inducer precursor, and hence

accounting for the more rapid phosphorylation in nitrate than ammonia grown plants. The presence of a powerful, non enzymic cytochrome C reductant(s) in chloroplasts isolated from tomato plants has so far prevented attempts to measure TPNH - Cytochrome C reductase activity. Whether this non-enzymic system is of any significance in vivo is not known.

Tentative evidence, Experiment XV, for the assimilation of $N^{15}O_3$ by chloroplasts isolated from nitrate grown, but not by chloroplasts from ammonia grown plants, points further to the possibility of nitrate inducing adaptive effects in the chloroplast.

Much further work is clearly needed on many aspects of this problem. Of particular interest would be to know whether the effect is confined to tomato plants growing in water cultures, or whether it can occur in other species and in plants growing in soils rather than in water cultures. Should the effect of nitrate versus ammonium nutrition prove to be more general, it would be of interest to examine it at the ecological level, in relation to the so-called "nitratophilous" plants.

S U M M A R Y

A study was made of the growth of young tomato plants (0 - 21 days from the time of expansion of the cotyledons) in water cultures. With a view to examining the influence of ascorbic acid on the assimilation of nitrate, the ascorbic acid content of plants receiving all the nitrogen supply in the form of nitrate or ammonia was investigated. Molybdenum deficiency reduced the ascorbic acid content of nitrate grown plants, deficiency symptoms were not obtained with plants receiving ammonium nitrogen.

In the course of this work it was found that plants grown with ammonia as the sole source of nitrogen do not produce as much growth as plants receiving nitrate as all or part of the nitrogen supply. The growth of plants receiving ammonia is characterised by a lower fresh and dry weight and a lower starch and lignin content than plants receiving nitrate. A divergence in the growth of nitrate and ammonia grown plants is observed in the shoots before the roots show any difference.

The ratio of inorganic to organic phosphate is much higher in ammonia than in nitrate grown plants. These observations suggested a difference in photosynthesis between nitrate and ammonia grown plants. Nitrate fed to ammonia grown plants brought about a rapid esterification of

phosphate in young leaves. Esterification was slower in mature, fully expanded leaves. The amount of phosphorylation occurring in ammonia grown plants following the feeding of nitrate might be accounted for by esterification coupled to the nitrate reduction. However, chloroplasts isolated from nitrate grown plants were able to carry out photosynthetic phosphorylation at four times the rate achieved in chloroplasts from ammonia grown plants, when no nitrate was present in the incubating medium.

This data is discussed in terms of a hypothesis concerning the electron transporting system of the chloroplasts, which might explain the difference in photosynthetic phosphorylation, and hence growth, between nitrate and ammonia grown plants. The hypothesis invokes the inductive formation of certain chloroplast components, nitrate functioning as the inducer. Tentative evidence for such a situation is provided by an experiment using $K^{15}O_3$.

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